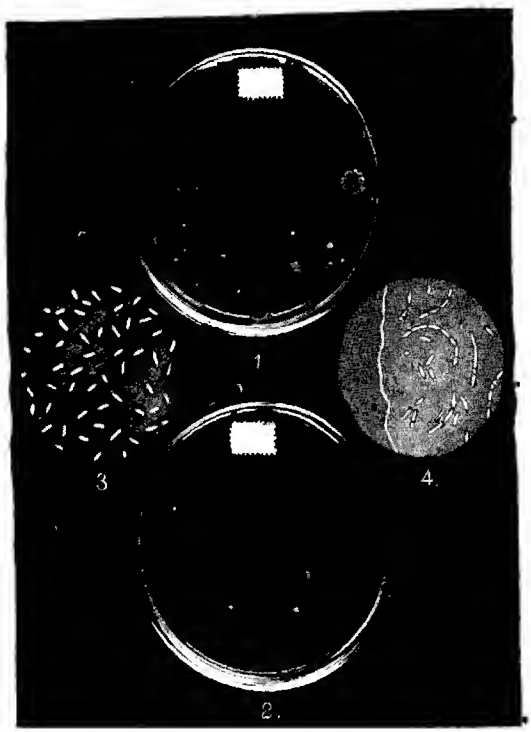
LABORATORY METHODS IN AGRICULTURAL BACTERIOLOGY



1. Germ content of air in cow-shed (§ 16). The Petri dish was exposed for $\frac{1}{2}$ min. and photographed five days later ($\frac{1}{2}$ natural size).
2. Germ content of air in laboratory (§ 16). The Petri dish was exposed for 30 mins. and photographed five days later ($\frac{1}{2}$ natural size).
3. Chinese ink preparation (§ 24). × 900.
4. Bacteria in hanging drop (§ 25). × 900.

LABORATORY METHODS IN AGRICULTURAL BACTERIOLOGY

BY

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AND REVISED BY THE AUTHOR.

WITH THREE PLATES AND FORTY FIGURES IN THE TEXT.

LONDON:
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EXETER STREET, STRAND.
1913.

AUTHOR'S PREFACE.

THERE is at present an abundance of literature dealing with practical bacteriology for medical students, and the same holds good with regard to microbiology in the brewery industries. On the other hand, there has always been a want of a laboratory book on methods of Practical Agricultural Bacteriology—Bacteriology of Foods, Dairy Products, Manures, and Soils. This book will, it is hoped, in some measure supply this felt want. We have been able to collect the material for this volume during our experience as teacher in the Agricultural Bacteriological Laboratory of the University of Leipzig.

The contents of the book have been so arranged that it should prove of value either as a guide to a complete course in Agricultural Bacteriology or to special work in some particular branch of the subject.

While the book has been designed specially for Students of Agriculture, we believe it will be found serviceable to others also. Thus Teachers of Agriculture, Agricultural Chemistry, and of other sciences relating to Agriculture, will probably find it useful when attempting to answer questions of a bacteriological nature which arise from time to time in their work.

Most of the experiments can be carried out with comparatively simple appliances. Those specially adapted for demonstration purposes are mentioned collectively on page 125, and could be made to illustrate lectures in class or at meetings of Agricultural Societies. Such experiments demonstrate the presence and activity of bacteria in a way which converts previously hazy notions into clear understanding.

Questions of a more special nature, which could not be adequately dealt with in this little book, have been taken up in detail in our *Handbuch der landwirtschaftlichen Bakteriologie*, where will also be found complete references. In response to requests from various quarters we have added in the Appendix, page 129, some remarks on the equipment of a bacteriological laboratory.

The illustrations are, for the sake of clearness, mostly in the form of sketches drawn by Herr A. Reichert after our directions. For the photograms we have to acknowledge indebtedness to Herr O. Schröter, lately assistant in the bacteriological laboratory.

F. LÖHNIS.

LEIPZIG.

TRANSLATORS' PREFACE.

BACTERIOLOGY is a science of recent growth, and Agricultural Bacteriology may be said to have all developed within the last twenty years. But at the present day the curriculum of an Agricultural or Dairy College is incomplete without a course in Bacteriology. Bacteriology, like Botany or Chemistry, cannot be satisfactorily taught in the lecture room alone. It is one of the present day requirements that each student of Agriculture or Dairying should work through a systematic course of Practical Bacteriology in the laboratory.

The author has pointed out that there was need of a laboratory book on Agricultural Bacteriology in Germany. It seemed to us that this want was even more felt in this country. We feel that no apology on our part is required for introducing an English edition of this little book on laboratory methods.

It was our good fortune to pursue our own studies in Practical Bacteriology in Professor Dr. Löhnis's laboratory in Leipzig. Dr. Löhnis is the author of the *Handbuch der landwirtschaftlichen Bakteriologie*, which is recognised in all countries as the standard modern work on Agricultural Bacteriology. Any one who comes into contact with Dr. Löhnis in his laboratory is very soon impressed by his skill and resource in technique. That his laboratory is known far beyond the confines of the German Empire is shown by the many nationalities represented among his students.

It was a happy inspiration on our part when we suggested that the book which he was then preparing should be translated into English, and our Publishers kindly

permitted us to defer the completion of a large work on Dairying so as to allow of the early issue of this handbook. Only the comparative few could find it convenient to study bacteriological methods in Leipzig, but, by means of this translation, Dr. Löhnis's methods could be brought within reach of all English readers. It is, as far as possible, a literal translation of the original, but has been revised and approved by the author, who reads English without difficulty. A few names have been added to the list of English and American books of reference and to the list of firms supplying reliable bacteriological apparatus.

We wish to acknowledge our indebtedness to Professor Dr. Löhnis for advice freely given at all times and for the trouble he has taken in reading and revising the English manuscript: and we also take this opportunity to acknowledge his invariable kindness and courtesy to us while working in his laboratory.

W. S.
J. H. S.

GLASGOW.

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INTRODUCTION.

Object and Scope of Practical Agricultural Bacteriology. In general terms laboratory work in Agricultural Bacteriology provides information with regard to the presence and activity of micro-organisms in food-stuffs, milk and its products, farmyard manure, and in soil. When a specific change or decomposition is being investigated, observations ought to be made in the following order:

(1) *Whether, by introducing a small quantity of the changing or decomposing material into a prepared medium of similar properties, the same effects can be reproduced.*

(2) *Whether micro-organisms are present, and, if present, which of them have been responsible for the change.*

(3) *Whether, and how, it is possible to inhibit an undesirable change, or promote a desirable one.*

Of course, it is often necessary to restrict the investigations to some particular point. For example, if a milk fault is to be suppressed, the main object will be to discover some remedy; but the result will be the more satisfactory the more completely the above three investigations are carried out.

Books of Reference.

For information on special points, the following works will be found useful:

1. Microscopical Technique.

Ehrlich-Weigert, Enzyklopädie der mikroskopischen Technik.
Hager-Mez, Das Mikroskop und seine Anwendung.
Strasburger, Das botanische Praktikum.

LABORATORY METHODS IN

GENERAL BACTERIOLOGICAL TECHNIQUE.

Günther, Einführung in das Studium der Bakteriologie.
Heim, Lehrbuch der Bakteriologie.
Hueppe, Die Methoden der Bakterienforschung.

BACTERIOLOGICAL DIAGNOSIS.

Chester, A Manual of Determinative Bacteriology.
Lehmann u. Neumann, Atlas und Grundriss der Bakteriologie.
Matzuschita, Bakteriologische Diagnostik.
Migula, System der Bakterien, Bd. II.

AGRICULTURAL BACTERIOLOGICAL TECHNIQUE AND DIAGNOSIS.

Lafar, Handbuch der technischen Mykologie, Bd. II u. III.
A Handbook on Technical Mycology; English translation by Salter. Vol. I.
Löhnis, Handbuch der landwirtschaftlichen Bakteriologie.
Percival, Agricultural Bacteriology, Theoretical and Practical.

METHODS IN BIOLOGICAL AND AGRICULTURAL CHEMISTRY.

Abderhalden, Handbuch der biochemischen Arbeitsmethoden.
Wiley, Principles and Practice of Agricultural Analysis: Vol. I. Soils; Vol. II. Fertilisers and Insecticides; Vol. III. Agricultural Products.

DAIRY BACTERIOLOGY AND METHODS OF EXAMINING DAIRY PRODUCTS.

Conn, Practical Dairy Bacteriology.
Kirchner, Handbuch der Milchwirtschaft.
Russell and Hastings, Experimental Dairy Bacteriology.
Sommerfeld, Handbuch der Milchkunde.
Teichert, Methoden zur Untersuchung von Milch und Molkereiprodukten.

MICROBIOLOGY OF FERMENTATIONS, YEASTS, AND MOULDS.

Henneberg, Gärungsbakteriologisches Praktikum, Betriebsuntersuchungen und Pilzkunde.
Jörgensen, Die Mikroorganismen der Gärungsindustrie.
Lafar, Handbuch der technischen Mykologie, Bd. IV. u. V.
A Handbook on Technical Mycology; English translation by Salter. Vol. II.
Lindner, Mikroskopische Betriebskontrolle in den Gärungsgewerben.

8. MICROPHOTOGRAPHY.

Barnard, Practical Photo-micrography.
Fuhrmann, Leitfaden der Mikrophotographie.
Neuhauss, Anleitung zur Mikrophotographie.
Neuhauss, Lehrbuch der Mikrophotographie.

9. RECORDS OF RESEARCH, JOURNALS, &C.

The amount of relevant literature is very considerable. The agricultural bacteriologist, in perusing several hundred Bulletins, Journals, Reports, &c., will occasionally find a few articles of interest. The chief results of research published up to the middle of 1909 will be found in the author's *Handbuch der landwirtschaftlichen Bakteriologie*. The most important work on agricultural bacteriology is the *Centralblatt für Bakteriologie, II. Abteilung* (containing results of original research and references to work done in general and agricultural bacteriology).

The following journals contain very complete accounts and references:

Centralblatt für Agricultur-Chemie, founded by R. Biedermann (partly detailed, and partly short references to works on agricultural chemistry and bacteriology). *Experiment Station Record*, an almost complete and world-wide summary of agricultural research. *Jahresbericht über die Fortschritte auf dem Gebiete der Agricultur-chemie*, founded by R. Hoffmann (with systematic references to publications on agricultural chemistry and bacteriology, and agricultural practice). *Jahresbericht über die Fortschritte in der Lehre von den Gärungsorganismen*, founded by A. Koch (with systematic references on general and agricultural bacteriology and the microbiology of fermentations).

Other journals may be mentioned:

Biochemisches Centralblatt (references on bio-chemistry of micro-organisms). *Bulletin de l'Institut Pasteur* (complete references for the whole field of bacteriology). *Centralblatt für Bakteriologie, I. Abteilung* (original works and references, principally on medical bacteriology). *Chemisches Centralblatt* (short references to the literature on agricultural chemistry and bacteriology, and generally issued very soon after the publication of the new work). *Journal of the Chemical Society* (besides original works, and references to work on pure chemistry, also references to works on agricultural chemistry and microbiochemistry).

A. INTRODUCTION TO BACTERIOLOGICAL TECHNIQUE.

EXPERIMENTS WITH AIR, WATER, AND FOOD-STUFFS.

I. APPARATUS. INSTRUMENTS. LABORATORY RULES.

1. **Apparatus.** The more important apparatus in the bacteriological laboratory includes:

A. For *sterilisation* of instruments, vessels, and culture media:

(1) Hot-air oven.
(2) Steam steriliser.
(3) Autoclave.

B. For *cultivation* of micro-organisms at specific temperatures: Incubators or Thermostats.

The **Hot-air Oven** (Fig. 1) is a double-walled iron chamber, preferably lined with asbestos, which can be heated by gas or spirit to a temperature of 160°–170° C., or even as high as 200° C.

It is used chiefly for sterilising glass-ware, as will be explained later.

The **Steam Steriliser** (Fig. 2) consists of a zinc or tin cylinder lined with asbestos or felt, with a copper water-cistern at the lower end, beneath a perforated plate. The lid is shaped like a flattened cone, has two hand knobs, and fits loosely into a groove. To increase the space for sterilising, a second cylinder of the same size can be placed above

the first. A constant water-level adjustment, though not indispensable, is of advantage as a safeguard against the water cistern becoming dry and being burnt through.

The Steam Steriliser is used chiefly for the preparation and sterilisation of culture media, as will be explained later.

The Autoclave (Fig. 3) consists of a strong cylindrical copper vessel the lower part of which is filled with water.

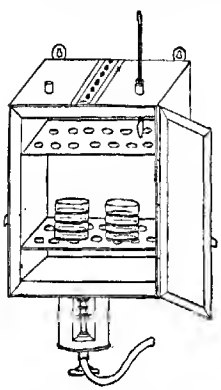


FIG. 1. Hot-air Oven (about $\frac{1}{15}$ natural size).

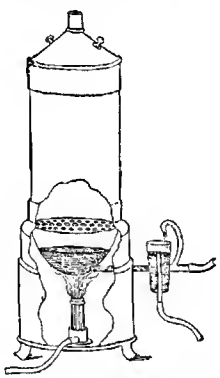


FIG. 2. Steam Steriliser with constant water-level (about $\frac{1}{15}$ natural size).

By means of a lever screw, the lid can be pressed down firmly on a leaden ring so as to make the apparatus quite air-tight. There are also a manometer or pressure-gauge and a safety valve, the latter having an opening or vent on one side which can be closed by a small screw. A case or socket is usually also provided in which a thermometer may be placed.

Particulars as to the use of the autoclave will be found in §§ 8 and 11. An autoclave is very desirable in certain cases to expedite

the werk, but it is quite possible to dispense with this rather expensive apparatus.

The **Incubator** or **Thermostat** (Figs. 4 and 5) is used for the purpose of securing a constant temperature in the inner space; and this end may be achieved in many different ways. As a rule, the apparatus takes the form of an oven of special construction, with a double jacket filled with water or glycerine, warmed by burners which automatically control the temperature. Should the flame become extinguished, the so-called safety burners prevent any escape of gas; for, on the temperature falling, the gas is automatically and completely shut off.

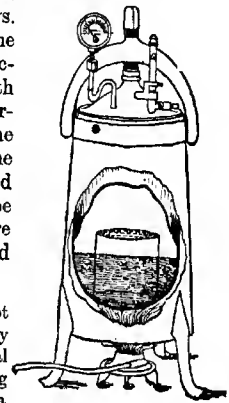


FIG. 3. Autoclave (about $\frac{1}{10}$ natural size).

The above-mentiened apparatus, except the autoclave, can be cheaply fitted up by any one pessessing ordinary mechanical skill. If necessary a pet containing warm or boiling water may serve as a thermostat or steriliser. If economy is net aimed at, special apparatus, illustrations ef which can be seen in the catalogues of the firms referred to in Appendix V, may be purchased. At the same time, in bacteriological work, comparatively goed results can be obtained by the use of quite simple apparatus.

In addition to such simple apparatus for the preparation of media as enamelled pots, measuring cylinders, tripods, Bunsen burners, wire baskets, &c., two specially constructed filter-funnels may be mentioned: the hot water filter and the filter apparatus for obtaining germ-free solutions.

The **Hot-water Filter** (Fig. 6) consists of an ordinary glass filter funnel fitting into a somewhat wider copper funnel of the same shape. The copper funnel has a tube projecting from near the bottom. Before the filter is used

the space between the two funnels is filled with water, which is heated by a burner placed under the projecting part.

The most important part of the **filter for obtaining germ-free liquids** is a porcelain or siliceous cylinder closed at one

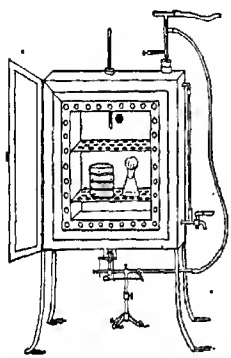


FIG. 4. Incubator with safety burner (about $\frac{1}{10}$ natural size).

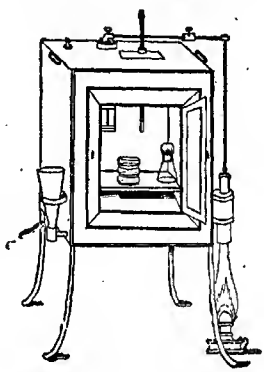


FIG. 5. Incubator, *after Sartorius* (about $\frac{1}{10}$ natural size).

end—the "filter candle." The liquid to be sterilised is sucked through the small pores of the filter by means of a water pump. The filtrate is collected in a sterilised chamber.

The details in the construction of these filters differ very much. An illustration of a comparatively simple type of filter candle (*after Maassen*) is shown in Fig. 7. The use of such a filter is restricted to the sterilisation of special media which would undergo undesirable alteration by the ordinary steaming methods (*compare* § 120).

2. **Apparatus.** The following is a list of the ordinary apparatus required. Instruments for special purposes are referred to later on in the text.

One microscope with a magnification of from 50–1000 (two

oculars, one with a micrometer, three objectives, triple nose-piece, large movable objective stage with Abbe's condenser) may, if necessary, be used conjointly by two students.

One platinum needle and one platinum loop, consisting of platinum wire 6–8 cm. long and 0·5 mm. diameter, fixed into a glass rod 20 cm. long and 6 mm. thick, for inoculating purposes.

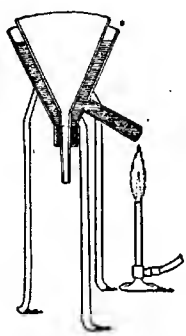


FIG. 6. Hot-water Filter ($\frac{1}{10}$ natural size).

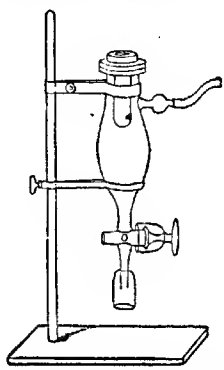


FIG. 7. Apparatus, *after Maassen* ($\frac{1}{10}$ natural size).

One Bunsen burner with bye-pass.

One pair fine forceps for use when washing cover glasses, &c.

One pair stronger forceps for drawing out plugs from anaerobic tubes, &c.

One pair Cornet forceps opening by pressure, for holding cover glasses when staining.

Thirty Petri dishes for plate cultures, with a diameter of 10 cm. and a height of 14 mm.

Two plates on which the Petri dishes may be placed and covered with bell jars.

Two dishes with lids (12–15 cm. diameter and 3–4 cm. high). A mixture of equal parts alcohol and xylol + about 3 per cent. hydrochloric acid is put into one. The other is reserved for clean object slides.

One large open dish (16 cm. × 7 cm.) into which specimens being stained are to be washed.

One wash bottle.

One small bottle with wide neck, glass rod, and glass cap, for cedar oil.

One similar bottle for Canada balsam.

Four ordinary bottles of about 100 c.c. capacity with corks, for stains, xylol, and acetic acid.

Five Erlenmeyer flasks of 300 c.c. capacity.

Twenty-five Erlenmeyer flasks of 50 c.c. capacity.

Two round flasks, one of 1000 c.c., the other of 2000 c.c. capacity.

One set of beakers (Jena glass with spout) of 1000, 800, 600, 500, 400, 300, 200 c.c. capacity respectively.

Two filter funnels, one large and one small.

Two hundred test tubes, 160 × 16 mm., for media, &c.

Twelve test tubes of thicker glass (same size) with rubber stoppers for anaerobic cultures.

Twelve large test tubes for fermentation and reduction tests, and holding pipettes, &c.

Six round glass discs, 13 cm. in diameter for anaerobic cultures.

Two stands, each for twenty-four test tubes.

Three boxes or glass jars for holding test tubes with prepared media.

One thermometer (to 120° C.).

Two glass rods.

Three Buri tubes (160 × 16 mm.) each with one rubber stopper, for anaerobic isolation.

One 9 c.c. pipette.

Ten 1 c.c. pipettes.

One pair scissors.

One horn spoon.

Flat and "hanging drop" object slides, 76 × 26 mm., thickness 1·2 mm., with ground edges.

Cover glasses, 18 × 18 mm., 0·15–0·18 mm. thick.

Labels, about 2 × 3 cm.

Filter paper.

Absorbent, and non-absorbent, cotton wool.

Soft cloth.

Glass pencil.

Pen and ink.

Note book.

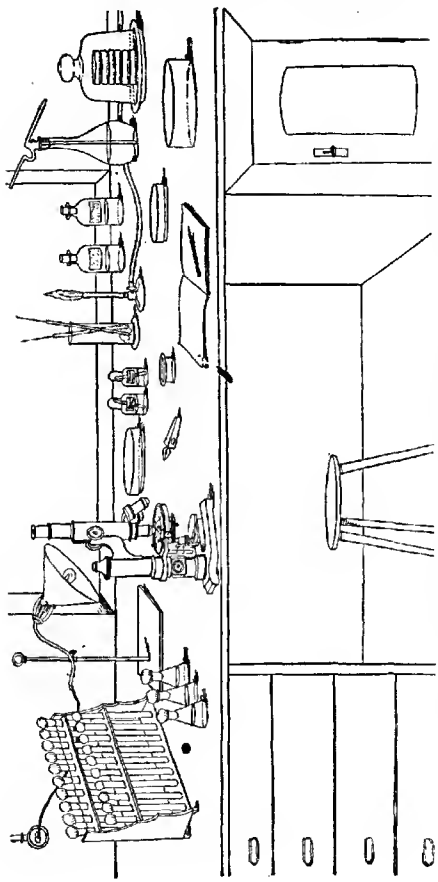


FIG. 8. Student's bench, showing apparatus (about $\frac{1}{15}$ natural size).

References. Details regarding apparatus will be found in the respective chapters in the works on bacteriological technique mentioned on p. 2. *See also:* Pfeffer, *Berichte d. deutschen botan. Gesellschaft* **13**, 1895, p. 49 (incubator room); Kuntze, *Centralbl. f. Bakt.*, II. Abt., **17**, p. 684 (large, comparatively cheap thermostat for 20° C.), also the illustrated catalogues of the firms for apparatus and instruments mentioned in Appendix V.

3. Laboratory Rules. For experimental work in Agricultural Bacteriology, experience in botanical and chemical (quantitative) methods is in every case of great advantage, and, when a thorough knowledge of the subject is wished, quite indispensable.

In order to save time in the laboratory, students are recommended to read the chapter or chapters in advance of their work. By so doing, not only will they be able to make the necessary preliminary preparations in good time, but they will be able to estimate the time required for the succeeding experiments and to arrange the work accordingly.

Special attention should be paid to the following Laboratory Rules, the respective reasons for which will be given later:

(1) *Observe the utmost neatness and cleanliness everywhere in the laboratory, and especially at the microscope table.*

(2) *Do not throw waste material (paper, cotton wool, matches, &c.) on the floor or into the sink. Place these in the waste box provided for the purpose. Remove from the laboratory at once all waste which is rapidly decomposing or readily attacked by moulds.*

(3) *Do not open media dishes rich in spore-bearing moulds incautiously. Avoid, as far as possible, raising dust or otherwise polluting the air in the laboratory.*

(4) *Clean thoroughly, and put back in their respective places immediately after use, such articles as are used by all the students in common,* i.e. *reagent bottles, measuring cylinders, wire baskets, &c.*

(5) *Handle the microscope with the greatest care; put it back in the microscope case immediately after use. After each use of the oil-immersion lens the objective should be carefully freed from cedar oil by means of a fine cloth moistened with a little xylol. Too much xylol must be avoided as xylol dissolves the mounting of the lens.*

(6) *When using the steam steriliser, make sure that there is enough*

water in the cistern, and that a sufficient water supply is maintained while in use. The leaden ring of the autoclave requires specially careful handling:

(7) *Dry all apparatus thoroughly before sterilising in the air oven. The oven must be allowed to cool before being opened.*

(8) *Do not open the incubator needlessly.*

(9) *Pour waste water containing acid into the special sink for that purpose.*

(10) *Avoid waste of gas and water. Before leaving the laboratory put out all gas flames except, of course, the incubator flames, which are kept burning constantly.*

II. CULTURE MEDIA.

4. **Standard Media.** A sufficient supply of the following six media should always be held in reserve for comparative experiments: *flesh gelatine, flesh agar, glucose agar, bouillon, milk,* and *potato slopes*. These media are well adapted for general purposes. Besides these, special media are often required in agricultural bacteriology. No organism, however, can be said to be fully described which has not been tested experimentally on the six standard media.

The different media are dealt with more fully in succeeding chapters. In the meantime, it will suffice to say that gelatine, from bones, is a nitrogenous medium, while agar, a carbohydrate from sea algæ, is almost free from nitrogen. Gelatine melts and solidifies at about 25° C.; agar melts at about 100° C. and solidifies at 40° C. The different properties of the two media determine which is to be used for any particular purpose.

5. **Cleaning of Test Tubes.** Put a certain number of test tubes (150) into a large pot with water, taking care to have as few air bubbles as possible remaining in the tubes. If the test tubes are new, add sufficient hydrochloric acid to give a distinct, but not strong, acid reaction; if the tubes have already been used, the addition of acid is not necessary. Place the pot on a tripod stand and bring the water to the boil by means of one or more burners. Then remove the flame and allow the water to cool. Finally, rinse the test

tubes thoroughly under the tap and put them to dry on the special stand for this purpose.

Water containing acid should be poured into the special sink for that purpose.

Hydrochloric acid is added in cleaning new test-tubes in order to neutralise the alkali yielded by new glass. If this were not done the reaction of the media filled into the tubes might be to some extent altered during sterilisation.

6. **Bouillon.** While the test tubes are being heated, prepare 1000 c.c. bouillon in one of the following three ways:

(*a*) Mix thoroughly one pound of lean, unsalted, minced meat with one litre of water. Allow to stand in a cool place for twelve to twenty-four hours. Filter through cloth, placed on a large filter funnel, into a 1000 c.c. measuring cylinder. To hasten the last filtering, bring together the ends of the cloth and press the flesh thoroughly until about 1000 c.c. filtrate is obtained. Make the volume up to 1000 c.c. and pour the whole into a large beaker. Add 1 per cent. peptone (Witte's) and ½ per cent. common salt. Place a flat glass dish over the beaker and heat for one hour in the steam steriliser.

Remove the extracted flesh from the laboratory at once.

(*b*) Heat 1000 c.c. tap water + 10 grms. Liebig's extract of meat + 10 grms. peptone (Witte's) + 5 grms. common salt in a covered beaker for one hour in the steam steriliser.

(*c*) Heat 22 grms. Ragit bouillon (from E. Merck in Darmstadt) + 1000 c.c. tap water, in a litre flask plugged with cotton wool, for one hour in the steam steriliser.

The first method (*a*) gives the most expensive bouillon, but for exact comparative work it is the best. Methods (*b*) and (*c*) are considerably cheaper, while (*c*) is to be recommended also for its simplicity.

When using the steam steriliser, make sure that there is enough water in the cistern and that a sufficient water supply is maintained while in use.

To remove any precipitate or insoluble matter, filter the hot bouillon through a large, folded filter paper. Test the

reaction and, if necessary, add concentrated soda solution carefully, drop by drop, till red litmus paper held in the hot bouillon is turned slightly blue.

Should the reaction become too alkaline, it must be corrected by the addition of phosphoric acid.

Another method of neutralising is as follows: Determine the exact amount of normal soda solution required to bring the 1000 c.c. of bouillon to the neutral point when phenolphthalein (1 c.c. 5 per cent. solution in 50 per cent. alcohol) is used as indicator and add 5–10 c.c. less than this quantity to the bouillon. Find this number of cubic centimetres NaOH in the following way: Boil 10 c.c. of the broth in a porcelain dish for three minutes and titrate, after adding a drop of the phenolphthalein solution, with N/10 NaOH till a faint, but distinct, pink colour is obtained. Multiply the result by 10 and subtract 5–10 from the total.

From the warm bouillon, measure out 700 c.c. for the preparation of flesh gelatine and flesh agar. Heat the remainder again for $\frac{1}{2}$–$\frac{3}{4}$ of an hour in a covered beaker in the steam steriliser and if necessary filter once more.

Before the measuring cylinder is used to measure any hot liquid, it should be warmed by adding slowly small quantities of the hot liquid. This will protect it from being cracked.

7. **Flesh Gelatine.** Add 10 per cent. of the best gold label gelatine to 350 c.c. of the filtered hot bouillon (§ 6). When this has dissolved, correct the reaction, which will probably be again acid, and heat in the steam steriliser for $\frac{3}{4}$ of an hour. Test the reaction once more and correct it if necessary. When the temperature has fallen to about 50° C., add the white of a *fresh* egg, which has been previously well stirred on a plate with a glass rod, and heat the gelatine for the last time in the steriliser for $\frac{1}{2}$–$\frac{3}{4}$ of an hour. Finally, filter the gelatine, through folded filter paper on the hot-water funnel (Fig. 6), into a beaker. If necessary, the filter paper may be doubled.

Clean the hot-water funnel immediately after use, using warm water to protect it from being cracked.

Properly prepared flesh gelatine ought to be quite clear, and solid

at the ordinary room temperature. If the above directions are followed closely, no trouble should be experienced in its preparation. It is difficult to obtain clear gelatine without the addition of albumen; *almost impossible if the reaction is too alkaline, if the egg used is* not quite fresh, or if any portion of the yolk of the egg gets into the gelatine. The filtered gelatine should never be left until the following day in an open beaker; moulds and bacteria would grow in it. The covered beaker, or the tubes filled with gelatine (*see* § 10), must be heated once more, on the same day, for ten to fifteen minutes.

8. **Flesh Agar.** Add 1½ per cent. of agar, in square lengths, to 350 c.c. bouillon. Before adding, reduce the agar to small pieces in order that it may dissolve more readily. Allow the mixture to soak for 1–2 hours, or overnight, in a cool place. Then heat it in the autoclave until the pressure is 1½ atms., or for 1 hour in the steam steriliser, and, finally, filter it through a fairly close plug of cotton wool, about 3 cm. in diameter, in the hot water filter funnel.

Rules to be observed when using the autoclave: First of all, see that there is sufficient water in the lower part. After the various articles to be sterilised have been placed in the apparatus, put the lid in its proper place. This is best done by turning it until the mark on the lid is in line with the mark on the under part. *Take care not to injure the lead ring.* Then screw the lid down tightly, see that the vent is left open, and light the gas. As soon as steam issues freely, close the vent. Continue heating until a pressure of 1½–2 atms. is registered on the pressure gauge. Then turn out the flame, and when the pressure has gone down to nil, open the vent slightly so that the pressure inside may gradually approximate to that of the air outside. A few minutes later, unscrew the lid and remove it carefully.

The more frequent mistakes made in the use of the autoclave are:

(1) *Closing the vent too soon.* A mixture of air and steam has much less sterilising power than pure steam.

(2) *Opening the vent too soon.* After the heating is over,

sudden reduction of pressure in the autoclave results in violent boiling over and spurting of the liquids which are being sterilised.

By means of a thermometer in its special place, the condition of the apparatus, and especially of the pressure gauge, can be tested from time to time. When steam is issuing freely from the open vent, the temperature ought to be between 95 and 98° C.; when the manometer registers 1 atm., the temperature should be about 120° C.; and when 2 atms., 130° C.

When pouring agar into the hot-water filter, press the cotton-wool plug gently with the glass rod down which the agar is poured. After filtering, clean the hot-water funnel as described in § 7. As in the case of gelatine, the filtered agar must also be heated once more on the same day.

The reaction, which has been already corrected in the bouillon, does not require to be tested after the addition of agar, since agar, unlike gelatine, does not affect the reaction in any way. Agar medium always remains more or less turbid.

Flesh agar can also be made direct by boiling in water ragit agar from E. Merck of Darmstadt (42 grms. + 1000 c.c. water).

9. **Glucose Agar.** Add ½ per cent. of glucose, which has been previously dissolved in a few c.c. of warm water, to 125 c.c. of the filtered flesh agar (§ 8).

10. **Filling the Media into Test Tubes.** In the spare intervals, while the flesh media are being prepared (*see* §§ 6–9), fit the clean, dry test tubes with suitable cotton-wool plugs. These plugs are best made from thin layers of cotton wool, taking pieces about the breadth of three fingers and the length of a finger. They should reach about 2–3 cm. into the tube, and above the mouth of the tubes have a diameter of 2–3 cm. In this way, the edges of the tubes are completely covered and the tubes properly closed (*compare* Figs. 9–11).

The plugs should be neither too loose nor too tight. While infection from the air must be made impossible, free passage of air should not be hindered.

Pour the filtered media into the test-tubes; any gelatine or agar which has become solid in the interval, place in the

steam steriliser for a short time, until melted. Pour about 8 c.c. of medium into each tube so that it occupies about one-third of the space below the plugs. There should now be, in all, about 40 flesh gelatine, 25 flesh agar, 15 glucose agar, and 35 bouillon tubes. Place these in wire baskets, with labels describing exactly the contents of each tube.

When gelatine or agar is being poured into tubes, the

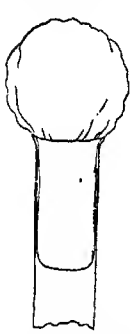


FIG. 9. Cotton-wool plug properly inserted.

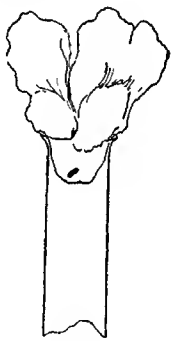


FIG. 10.

FIG. 11.

Cotton-wool plugs badly inserted.

liquid ought never to touch the upper part of the tube, as this would make the cotton-wool plugs adhere to the glass. Special apparatus for filling test tubes can be had, but such can be quite well dispensed with. With a little practice, the student will acquire the necessary skill. Hold the tube in an upright position, and pour the medium from a spouted beaker not more than half full. If it is found later that the cotton wool is sticking, the following rule should be observed:

Do not draw the plugs straight out, but first turn them round in the mouth of the tubes.

11. Sterilising the Flesh Media. Any germs present in the media must now be destroyed. To do this heat the bouillon and the gelatine in the steam steriliser for 15 min., on each of three successive days, commencing on the day when the medium is filled into the tubes.

The object of this "intermittent" sterilisation is to insure the destruction of spores. By the first heating, the spores are not killed, but in the intervals the conditions are favourable for their developing into the vegetative state, and in this form they will be destroyed by the second or third heating.

Do not put the wire basket with the tubes into the steriliser before the boiling temperature is reached.

The cotton-wool plugs should always be covered with a piece of parchment-paper in order that the water condensing on the inside of the steriliser lid may not drop upon them and make them moist.

On wet plugs, spores of moulds from the air may grow, their mycelium traverse the plug and produce spores on the under-surface. These spores naturally fall on the media and produce an abundant growth of mould.

If the room temperature is comparatively high, the gelatine must be kept in a cooler place, such as an insulated air- or ice-chamber.

Gelatine which has been heated too long, or kept in too warm a place, loses its power of solidifying.

Heat the flesh agar and the glucose agar once only, in the autoclave under a pressure up to 2 atms. For rules to be observed when using the autoclave, see § 8.

One heating in the autoclave is sufficient because a temperature of 130° C. kills spores. When sterilising, the temperature should never be higher than in preparing the nutrients, otherwise further precipitations take place. If no autoclave is available, proceed as with gelatine.

Allow the glucose agar and 15 of the flesh agar tubes to solidify in upright tubes. Place the other 10 agar tubes

in a sloping position, with the top end resting on a glass rod or piece of wood, &c. (*see* Fig. 12). The agar in this case solidifies in the form of a slope, which provides a large surface for streak cultures (§ 29). On solidifying, the agar gives up some water, which collects as water of condensation on the lower part of the tube.

12. **Milk.** Fill about 8 c.c. of clean, skim milk into each of ten test tubes, and sterilise them for ½ an hour in the steam steriliser on each of three successive days. The rest of the milk may be sterilised in a sufficiently large flask. It should be noted that a larger bulk, since the heat penetrates more slowly, must be heated for a correspondingly longer time. As a rule, the completely sterilised milk has a brownish colour, owing to the heating having changed the milk albuminoids and caramelised the milk sugar.

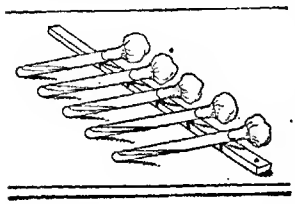


FIG. 12. Solidifying agar in slopes.

Dirty milk can be made sterile only by frequent and long heating; and sometimes a sterile product is never obtained. The necessary quantity of skim milk may be best obtained by starting with good, clean, whole milk and removing the cream by setting or centrifuging.

13. **Potato Slopes.** Take two or three large healthy potatoes and wash them thoroughly under the tap. Remove the skins evenly with a pocket knife. Cut them into wedge-shaped portions which will go easily into a test tube, and put into a beaker of water. Put a short piece of glass tube into each of ten test tubes, and add a little water from the wash bottle to prevent the potato wedge from becoming too dry during sterilisation. The potato should not touch the water (Fig. 13).

Suitable sized cylinders may be cut from the potato by means of a cork borer, and the cylinders cut diagonally, each half being put into a test-tube.

Sterilise the potato slopes in the autoclave (2 atms.). If no autoclave is available, treat them in a similar way to milk (§ 12).

14. **Preservation of Media.** Keep the test tubes which have been filled with the media in the special vessels reserved for that purpose. Label each vessel, stating the kind of medium and the date of preparation.

The wire baskets must always be emptied immediately after the sterilising.

FIG. 13. Potato slope.

Avoid keeping vessels containing tubes with medium in a hot place, or exposing them to sunlight. Drying of the medium can be prevented by moistening the cotton-wool plugs with a few drops of 1/1000 corrosive sublimate and fitting each tube with a suitable rubber cap, immediately after the last sterilisation. Sometimes it may be practicable to prepare larger quantities of the different media in open bottles with a spring and cap arrangement (as in milk and beer bottles) covered with a paper cap. After sterilising, close the bottles without removing the cap. However it is, as a rule, not advantageous to have too much in store. Freshly prepared medium always yields the best results.

References for §§ 4–14. The corresponding chapters in the works quoted on p. 2 and also in the *Grundriss der Bakteriologie* by Lehmann und Neumann. Ragit medium is recommended by E. Marx (*Münchn. mediz. Wochenschr.*, 1910, p. 361).

III. BACTERIA IN AIR.

Enumeration of Germs. Forms of Colonies.

15. **Preliminary Work.** Sterilise two Petri dishes in the air oven by heating them to a temperature of 165°–170° C. and allowing them to remain at this temperature for about five minutes. When the oven is cold take out the dishes and place them on the bench. Melt two sterile [1] gelatine

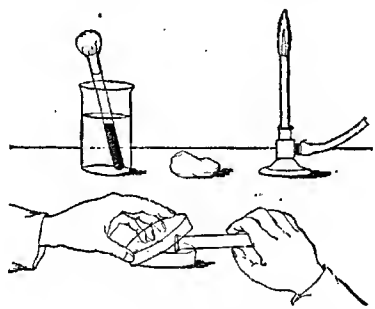


FIG. 14. Pouring gelatine into Petri dish.

tubes in a beaker with water at 40° C. Light the Bunsen burner and place it near the Petri dishes.

Now hold one of the gelatine tubes in an almost horizontal position, twist out the cotton plug, heat round the mouth of the tube by passing it through the flame, raise the lid of the Petri dish with the hand that is free just sufficiently to admit the mouth of the tube, and pour the gelatine into the Petri dish (Fig. 14). Put the lid again in position, and, by moving the dish, carefully distribute the gelatine

[1] For this particular experiment gelatine which has been heated only once in the steam steriliser can be used; so that it is not necessary to wait till all the gelatine has been thoroughly sterilised.

equally over the surface. Pour the second gelatine tube in exactly the same way, and allow the two plates to rest on a level surface till the gelatine has solidified.

In summer, gelatine plates should be placed on a surface which is cooled from below with cold water or ice. The gelatine must be of a uniform depth so that, over the whole plate, the bacteria can grow under approximately similar conditions.

The emptied test tubes are kept in a special vessel. When a sufficient number have been collected, they are boiled and cleaned before being used again. The cotton-wool plugs, if not too dirty, are also put aside for future use.

16. **Catching the Bacteria from the Air.** When the gelatine has solidified, bacteria from the air are secured by raising the lid of the Petri dish (which should not be turned over) and exposing the gelatine surface for a certain time to the atmosphere. For example, one of the plates might be exposed for 5–20 minutes in the laboratory, the air of which is comparatively free from bacteria, and the other for ½–2 minutes to the air of the cow-shed, where many more germs are present. At the end of the exposure the plates are again closed and a label giving the particulars is fixed on each a short distance from the middle. Both Petri dishes are then put on a porcelain plate covered with a bell jar, but not in a hot place, or directly in the sunshine.

The plate and bell jar may be moistened with cotton wool which has been dipped in 1/1000 corrosive sublimate (to secure sterilisation and a moist atmosphere). Gelatine plates should be lifted by the sides only, as the warm finger on the bottom might melt the gelatine.

The germs which fall on the gelatine develop distinct colonies in 1 to 10 days, according as the medium proves suitable for their growth (Plate I., Figs. 1 and 2).

Of course, by this method only a proportion of the bacteria in the air are examined, but for *approximate*, practical purposes

the method is quite satisfactory. The testing of the influence of the bacteria in air on milk is dealt with in § 57.

17. **Numbering of Germs.** The number of colonies would represent the exact number of germs if the following two conditions were fulfilled:

(1) If all the germs which fell on the medium developed colonies.

(2) If each colony originated from one germ only.

But, as a matter of fact, these two conditions are never exactly fulfilled. On the one hand, a greater or smaller number of germs do not grow because of unfavourable conditions—the medium may not be suitable, there may be too much or too little air, or the temperature may be too high or too low, etc.; on the other hand, it is frequently found on investigation that one colony contains not one species only but different sorts of bacteria. This latter circumstance might be caused, for example, by a particle of dust falling from the air. The bacteria adhering to it would be fixed in the same place and would form a mixed colony.

Hence it is only permissible to conclude that a certain number of germs has been found by a particular method, but not that this was the exact number actually present.

The very great accuracy in the counting of colonies which is sometimes expected seems, therefore, not to be absolutely essential. For comparative figures the gelatine plates should be kept for eight to ten days. Colonies of moulds and of liquefying bacteria may be killed by lightly touching each with a stick of silver nitrate (method recommended by Hiltner and Störmer). This should be done while the colonies are still quite small. Otherwise liquefaction may be continued by the liquefying enzymes already formed, or moulds may be further spread by spores thrown off by touching with the silver nitrate stick.

During the "killing" operation the lid of the Petri dish should be raised only so far as is necessary. Another method of performing

this operation is as follows: the Petri dish is held in a vertical position, and the lid removed and placed on the table. At the same time the under half of the dish is quickly turned upside down. In this way the colonies can be conveniently observed from above, while from below they are touched with the silver nitrate stick. Where several students are working at the same time on the same material, some of them should kill the colonies early, while others might allow the growth to proceed unhindered. In the latter case, especially with plates exposed in the cowshed, rapid liquefaction of the gelatine, associated with putrefactive odours, would probably result.

Special plate counters, formerly in common use, can, for the above reasons, be dispensed with. Sufficient accuracy is obtained by turning the plates upside down and, on the under side, marking the colonies with ink points.

18. **Form of Colonies.** Plates thickly overgrown with colonies give a fine illustration of the varied forms and colours which may be assumed. With a glass-writing pencil mark different colonies with numbers or letters and observe their development from day to day. Keep a special note-book with a record of the observations made, and make rough sketches of the appearance of the colonies as seen by the naked eye (macroscopic), and also under the low power of the microscope (without Abbe's condenser). By the unaided eye the shape and optical properties can be seen; while the microscopic examination shows the interior structure and gives a distinct view of the edge of the colonies (*compare* Figs. 15–18).

Observe especially whether the colonies are transparent, translucent, or opaque; iridescent, opalescent, or more or less radiant; chalky or porcelain-like; uniformly coloured throughout or not; whether the liquefied portion of the gelatine is clear or uniformly turbid, or whether the colony, during liquefaction, breaks into more or less large pieces, presenting a crumbly appearance; whether, on touching the colony with the platinum needle, it shows a watery, slimy, thready, or cartilaginous consistency; or if it can be lifted from the medium as a tenacious film; and whether the colony possesses any other distinctive features.

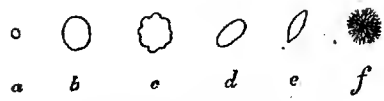


FIG. 15. Form of Colonies.
(*a*) round, (*b*) discoid, (*c*) irregular, (*d*) elliptical, (*e*) lentiform, (*f*) ramified.

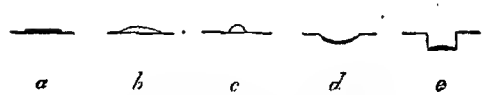


FIG. 16. Elevation of Colonies.
(*a*) flat, (*b*) convex, (*c*) droplike, (*d*) concave, (*e*) annulate depression.

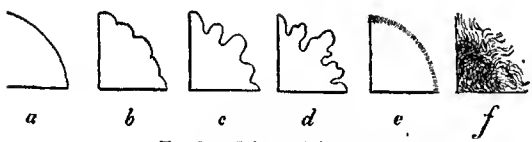


FIG. 17. Edge of Colonies.
(*a*) entire, (*b*) crenate, (*c*) lobate, (*d*) auriculate, (*e*) ciliate, (*f*) curled.

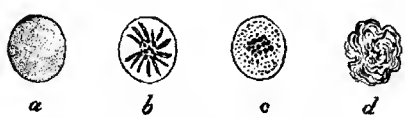


FIG. 18. Structure of Colonies.
(*a*) granulated, (*b*) with radial stripes, (*c*) spotted, (*d*) wrinkled.

IV. BACTERIA IN WATER. ISOLATING BACTERIA.

19. **Preliminary Work.** Put 9 c.c. of tap water into each of two test tubes and close the tubes with cotton-wool plugs. Place three 1 c.c. pipettes in a large tube, also closed with cotton wool. It does not matter if the top ends of the pipettes project a little way through the cotton wool. A cap of parchment paper can be placed over the projecting ends, to afford some protection against infection. Heat the tube with the pipettes, and the two test tubes with water, in the autoclave to 1½ atms., or in the steam steriliser for two hours.[1] At the same time sterilise three Petri dishes and a beaker in the air oven. If tap water is to be examined, allow the water to run from the tap for some time in order to get a fair sample, which is finally run into the sterilised beaker. The distilled water which is in use in the laboratory might also be examined.

Good tap water contains, as a rule, very few bacteria; but in many cases the number present may be considerable (distilled water kept for some time in the laboratory, water for use in the dairy, stable, &c.). In these latter cases it is necessary to fill a larger number of tubes with 9 c.c. water and to sterilise a correspondingly greater number of 1 c.c. pipettes, so that greater dilutions may be made. The method of taking the sample depends upon circumstances; the water sample should be protected from foreign infection and kept in a cool place until all preliminary preparations have been made.

20. **The Making of Dilutions and Plate Cultures.** Melt three gelatine tubes in a beaker with warm water. Take 1 c.c. of the water to be examined, by means of one of the sterilised pipettes, and run it into the test tube marked I—1st Dilution. Immediately thereafter transfer a second 1 c.c. to a Petri dish, raising the lid only so far as is necessary. Mark this plate O—Original. The tube to

[1] If pipettes are repeatedly sterilised in the air oven, the glass will, in a comparatively short time, be injured.

which 1 c.c. of the water has been added should be at once closed with its cotton-wool plug and then shaken vigorously. With the second sterile pipette transfer 1 c.c. from the first dilution to test tube II. and 1 c.c. to the second Petri dish (1st Dilution = 1/10 Original). In the same way from test tube II., after vigorous shaking, remove 1 c.c. into the third Petri dish (2nd Dilution = 1/100 Original). Now empty a gelatine tube into each Petri dish and mix the gelatine and the water thoroughly and carefully. When the gelatine has solidified place the plates under the bell jar, and examine them in two to four days, or as soon as colonies are visible, as directed in §§ 17 and 18.

For exact work duplicate plates are essential. In the method of examination of anaerobic bacteria the water is brought into three melted agar tubes, which have been cooled to 50° C. By a gentle to-and-fro motion, not by shaking, a proper mixture is obtained and further investigations made as explained in § 41. The special examination of water for dairy purposes is dealt with in §§ 57 and 58.

21. **Isolating Bacteria.** The colonies which are obtained direct from air and water are often not from one germ, but are *mixed cultures*. *Pure cultures* are obtained in the following way:

From each colony which it is desired to study more closely (for example, two from the air and two from the water plates), first inoculate a tube of bouillon. To sterilise the platinum needle for this purpose, hold the glass rod with the wire in the flame, and pointing downwards, till it becomes red hot; then move the adjoining part (about 10 cm.) of the glass rod through the flame, holding it horizontally and moving it backwards and forwards. Then allow to cool, with the needle pointing upwards, for twenty to thirty seconds. Now take a tube of bouillon, hold it in as horizontal a position as possible, and remove the cotton plug by the second and third fingers of the right hand in such a way that the lower part of the plug going into the tube projects outwards between the two fingers. Touch the colony with the point

of the needle, and transfer a very small (almost invisible) quantity to the bouillon tube, the mouth of which has been just previously heated in the flame. The infection of the medium is secured by rubbing the needle against the side of the tube just above the liquid bouillon. Again heat the mouth of the tube in the flame, insert the plug, and sterilise the needle thoroughly as before. Label the bouillon tubes and keep them for one to two days till a distinct turbidity or a considerable precipitato becomes evident.

Always heat the platinum wire of the needle or of the loop till it becomes red hot throughout its whole length immediately before and after use. Also cautiously sterilise in the flame the adjoining part of the glass rod so far as it reaches into the culture dishes and tubes.

All culture tubes when opened should be held as nearly horizontal as possible. Risk of foreign infection is further minimised by keeping the air in the laboratory as still and as free from germs as possible. Bacteria and moulds attached to the mouth of the tube should be destroyed by heating this part before and after inoculation.

Three sterile plates, and three gelatine tubes in a beaker of warm water, are required for each bouillon culturo which has produced a satisfactory growth. Sterilise the platinum needle and hold it, pointing upwards and outwards, between the forefinger and the middle finger of the right hand. Take the bouillon tube, which has been previously shaken, between the forefinger and the middle finger, and one of the melted gelatine tubes between the thumb and forefinger of the left hand, both in as horizontal a position as possible. Fix the plug of the gelatine tube between the middle and third fingers, that of the bouillon tube between the third and fourth fingers of the left hand, in such a way that the parts of the plugs which enter the tubes project freely outwards. This prevents infection from the fingers. After heating the open ends of both tubes in the flame, insert the platinum needle to the bottom of the bouillon, keep it there for a few seconds, then quickly transfer it to the gelatine and stir the latter round

to produce a thorough infection (Fig. 19). Heat again the open ends of the tubes in the flame, insert the plugs in their respective tubes, heat the platinum needle, and put the bouillon aside. Next carefully sterilise the platinum loop and make the next dilution in exactly the same way. Place the newly inoculated gelatine tube between the forefinger and the middle finger, a fresh one between the thumb and forefinger, and fix the plugs in their places as before. Transfer

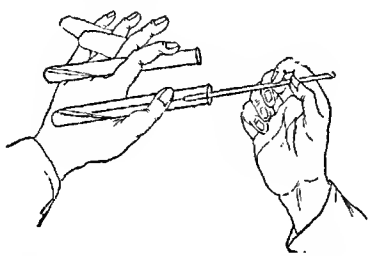


FIG. 19. Inoculating, and making dilutions in test-tubes.

a loopful of the infected gelatine, 1st Dilution, into the second tube of gelatine, 2nd Dilution. The liquid on the loop is readily removed by lightly tapping the side of the tube just above the surface of the gelatine, and a thorough mixture is obtained by stirring thereafter with the platinum loop. Then plug the tubes and sterilise the platinum loop thoroughly. When Dilution III. has been made in the same way, pour the three tubes, after heating the open ends, into three Petri dishes properly labelled.

After a little practice, the process of inoculating and making of dilutions will present no difficulty. It is much simpler than it will appear on first reading these directions. The mixing of the germs in the gelatine must not be done by vigorous shaking, as air bubbles would appear in the plate cultures.

The plates are kept in the usual way and examined later according to the instructions given in § 18. In a really pure culture all colonies growing on the surface have a quite similar appearance. The same applies to colonies lying inside the gelatine and also to those fixed next to the glass at the bottom of the plate.

Deep-seated colonies of most species of bacteria remain, as a rule, very small and have a very similar appearance. Generally they are quite different from the colonies growing on the surface. Colonies, again, at the very bottom of the medium are more like surface colonies; but such bottom colonies are comparatively few in number.

When the colonies are sufficiently developed, select one well apart from the others, and inoculate from it a bouillon tube. This bouillon culture is to be used later for microscopic and cultural tests (Ch. V. and VI.).

Used Petri dishes are readily cleaned by first placing them, both halves with the flat side downwards, in a pot of water, boiling the water, allowing it to cool, and then thoroughly rinsing the dishes under the tap.

V. MICROSCOPIC EXAMINATION.

22. **Preparation of Stained Specimens.** Dry some glass slides and cover glasses, taken from a dish half filled with a special cleaning mixture of alcohol and xylol and about 3 per cent. hydrochloric acid, with a soft, non-fibrous cloth, and polish them till quite clear. The cover of the xylol-alcohol dish must be replaced immediately. Lay the clean slides and cover glasses on filter paper or in a clean vessel.

Prepare stained specimens as follows:

1. Transfer a loopful from one of the bouillon cultures, in the usual careful manner, to a glass slide lying on the bench. *Spread out* the drop of material so that it occupies about 1 sq. cm.

2. In a short time the smear becomes *air dry*. If necessary, hasten drying by warming very gently over a small flame.

3. Hold the slide with the smeared side uppermost, taking the edges at the end between the thumb and forefinger, and *fix* the bacteria by moving the slide slowly, about one second each time, four or five times through the hottest part of the flame.

4. *Stain* the bacteria by flooding the smeared part for about thirty seconds with an aqueous solution of an aniline dye—Victoria blue.

5. Then pour off the stain and *wash* the preparation thoroughly with water from the tap or the wash-bottle.

6. *Dry* the slide first with filter-paper and then by gently warming over a small flame.

7. The specimen may be preserved by *mounting* in Canada balsam. Place a drop of balsam, about 3 mm. in diameter, in the middle of the smear and cover it with a cover glass. The Canada balsam bottle should not be left open.

Canada balsam, a solution of coniferous gum in xylol, should not have an acid reaction. If it is acid some powdered sodium carbonate should be added, and the mixture kept in a warm place for some time and frequently shaken. The carbonate may be finally separated from the balsam by allowing it to settle out.

Any air bubbles in the drop of balsam on the slide can be removed by touching them with a hot platinum needle before covering.

Canada balsam will generally spread itself uniformly; but, if it does not, spreading can be effected by gently warming it over a small flame. If the drop is of the proper size the space between the glass slide and the cover glass will be exactly filled; but if it is too large the excess of balsam will ooze out beyond the edges of the cover glass. This latter contingency should be carefully avoided.

The aqueous aniline stain is prepared from a saturated alcoholic solution of the dye. The dry colouring matter is added to absolute alcohol till a portion remains undissolved. This concentrated

solution is diluted with about ten times its volume of water, in small bottles. The diluted solution soon spoils and must be frequently renewed and filled each time into clean bottles. For our present purpose only two out of a large number of stains require to be considered, *e.g.* Victoria blue and methylene blue. The latter is a less intensive stain than the former.

Bacteria differ in their **staining** properties. The time stated above for staining must sometimes be increased, or special methods, which will be described later, adopted.

Should it be found on examination (*see* § 23) that the stained preparation is not quite satisfactory (as may naturally be the case with first attempts), place it in the alcohol-xylol mixture, which in a short time dissolves the balsam and removes the stain. After a few trials good specimens should be readily obtained, *i.e.* specimens which are **as free as possible from foreign matter,** contain **neither too many nor too few bacteria,** and in which the bacteria are **uniformly distributed and well stained** (Fig. 20).

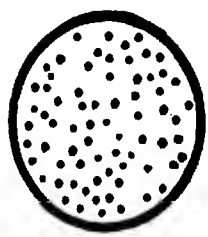


FIG. 20. Stained specimen; air bacteria × 1200.

The mounting in Canada balsam may be deferred till it is found whether the specimen is worth keeping. The cedar oil is readily removed with xylol (*see* § 23). When specimens are to be kept, they should be properly labelled. Canada balsam becomes quite solid after a time, but it can be dissolved whenever desired with xylol. In this way old faded specimens can be thoroughly cleaned with xylol and freshly stained. If slides are to be kept for some time, a case for their reception is to be recommended.

23. **Use of Oil Immersion Lens.** For the examination of bacteriological specimens a magnifying power of 600–1000 is necessary. Good pictures by the dry system can be got only by the use of special, rather expensive lenses—the so-called apochromatic lenses. As a rule, the

oil immersion lens is used, which, in conjunction with *Abbe's condenser*, gives a specially clear and sharp picture. Cedar oil, which forms the medium between the specimen and the objective, has the same refractive index as glass. The rays of light passing through the specimen are hence more nearly parallel than in the dry system (*see* Fig. 21).

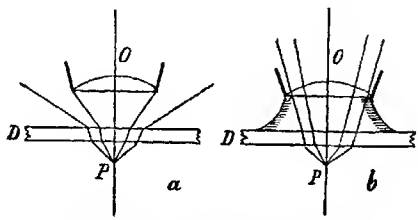


FIG. 21. Refraction of rays of light (*a*) in dry system, (*b*) in oil immersion system, from specimen P, through the cover-glass D, in the objective O.

The oil immersion lens should be used in the following way:

1. Place a drop of cedar oil, about 3 mm. in diameter, on the cover glass, or directly on the dried smear. The cedar-oil bottle should be *promptly* restoppered.

2. Place the specimen in the middle of the microscope stage and lower the tube of the microscope by the coarse adjustment till the immersion lens touches the drop of oil.

3. Lower the microscope tube further very slowly and cautiously, moving the specimen gently at the same time, and observe when the lens just touches the specimen very lightly so as to hinder the movement of the slide.

The lens must on no account be pressed against the cover glass.

4. Adjust the mirror and diaphragm till the field of vision is as clear as possible.

The flat mirror is used in daylight and the concave one with artificial light.

5. Raise the microscope tube very gradually (the screw ought to be turned only a fraction of a millimetre per second), moving the specimen continually (objects in motion are more readily detected).

6. As soon as the bacteria become visible, use the fine adjustment to get sharp focus.

7. If necessary, readjust the diaphragm and regulate the light. Instead of moving the slide directly, it is now better to move the stage, if the microscope is provided with a movable one.

The student should become accustomed, when making observations, to keeping the objects constantly in focus with the fine adjustment. The eye not in use should not be closed tightly.

The ocular or eye-piece must never be opened, except when absolutely necessary, and then only with the greatest caution. The objective must on no account be unscrewed.

Further information with regard to the theory and use of the microscope will be found in the special chapters in the books on the microscope referred to on p. 1. Experience teaches best.

From specimens which are to be kept the bulk of the oil is removed with filter paper, and the remainder is allowed to dry. When examining an old specimen the dried oil is removed with xylol before a fresh drop of oil is placed on the glass.

After each use of the oil-immersion lens the objective should be carefully freed from cedar oil by means of a fine cloth moistened with a little xylol. Too much xylol must be avoided, as xylol dissolves the lens mounting.

Put the microscope back into its case immediately after use.

24. **Chinese Ink Preparations.** The staining of bacteria is a common and most useful method of studying bacteria, as the direct examination of the living organisms, especially when they are motile, presents considerable difficulty. For this reason the methods of observing living bacteria have not yet been described (*see* § 25). Objections to stained

specimens, however, are that the shape and structure of the organisms are more or less altered by the manipulations —fixing, staining, &c. The examination of unstained specimens is only in certain cases applicable (*compare* § 88). The unstained bacteria can, however, be advantageously examined when they are embedded in Chinese ink. They are then seen as clear spots against a dark background (*see* Plate I. Fig. 3). This preparation is made by mixing, as uniformly as possible, on the glass slide a loopful of sterile Chinese ink [1] (from Grübler & Co., of Leipzig) with a drop of liquid containing the bacteria.

In a short time the smear will be dry, and, properly prepared, it presents a dark grey appearance when held against a white background. The mounting in Canada balsam is done as in the case of stained specimens, but the specimen can also be examined direct with the oil-immersion lens without a cover glass. Between parts which are too dense and parts too thin places are nearly always to be found affording a very clear picture. The method is to be specially recommended for microscopic examination of bouillon cultures, milk, etc., which, owing to the presence of foreign matter, are not so well adapted for staining. This Chinese ink method is not so successful when the material containing the bacteria is very slimy.

Reference. Burri, *Das Tuscheverfahren*, 1909.

25. **Observation with the Hanging Drop.** Lay a slightly greasy cover glass on the bench and place a loopful of the bouillon culture upon it so as to form a flat droplet about 2–3 mm. in diameter. The greasiness prevents the drop spreading too much; this condition may be obtained,

[1] Ordinary Chinese ink usually contains bacteria. It must therefore be sterilised in the steam steriliser and any sediment allowed to settle out till a homogeneous liquid with only the finest particles in suspension is obtained. The loopful must always be taken under aseptic conditions; and the ink should be from time to time diluted with water and again sterilised.

if necessary, by lightly touching the glass with the finger. Round the hollow part of the slide (Fig. 22) place four small spots of Canada balsam or vaseline. Turn the slide upside down and lower it carefully on to the cover glass, the corners of which should touch the balsam or vaseline spots. This

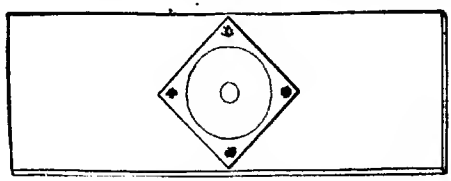


FIG. 22. Slide with ground hollow, showing a hanging drop. Natural size.

keeps it in position. The drop of liquid remains free in the hollow space (Fig. 22).

If the drop is too large and touches the slide, microscopic examination is not possible. If too much balsam or vaseline is used air is completely excluded from the hollow in the slide and motile bacteria soon lose their motility.

Now turn the slide over quickly so that the droplet does not change its position, and put a drop of cedar oil on the cover glass. Lower the tube of the microscope as before (§ 23), but take special care that the objective is not pressed through the cover glass. It is best to use artificial light—electric or gas—and the concave mirror. First bring the full light to bear on the specimen, then close the diaphragm till the field of vision appears light grey. Now raise the microscope tube very slowly, moving the specimen continually. As soon as the bacteria are seen use the fine adjustment until sharp focussing is obtained. Keeping the objects constantly in focus, with the micrometer screw move the slide sideways till the edge of the drop comes into view, as it is there the bacteria are best seen (Plate I., Fig. 4), provided the light and diaphragm are properly adjusted.

Notice whether the bacteria are actively motile or not. Towards the centre of the drop all bacteria, as a rule, appear motile, owing to the so-called **Brownian or molecular movement.** By this movement not only the single bacteria but also conglomerates of them are swinging and dancing in a characteristic manner, but no progressive advance can be observed. Really motile bacteria are to be seen here and there at the edge of the drop in more or less rapid progressive movement, and it is only in comparatively few cases with sluggish bacteria that any doubt can arise.

The hanging-drop observations require considerably more patience and practice than staining or making Chinese ink preparations. If it is desired to make a fresh hanging drop, raise the cover glass carefully (with the forceps) by holding it at the upper and lower corners. The slide is then free for further use; the cover glass is put into the xylol-alcohol mixture.

26. **Measuring of Bacteria.** Make a stained or a hanging drop preparation in the usual way and observe the bacteria through a special eye-piece fitted with a micrometer. The length and breadth of the bacteria are reckoned in micrometer spaces, and the actual dimensions calculated from the scale provided with each microscope.

When, for example, the length of the bacteria corresponds to 1-1½, and the breadth to half a micrometer space, and one micrometer space equals, say, 1·65 μ the actual measurements will be $0{\cdot}8 \times 1{\cdot}65\text{-}2{\cdot}5\,\mu$. Of course, in order to get the true or average dimensions a large number of bacteria must be measured. The stained bacteria, on account of shrinkage due to drying and fixing, have, as a rule, smaller dimensions than the living organisms. For this reason measurements must be made of both living and stained specimens.

27. **Staining of Flagella.** Satisfactory staining of flagella requires, as a rule, considerable time and is often very difficult. In Agricultural Bacteriology it is necessary to make such specimens only when an exact investigation of an unknown species has to be made. A large number of methods are already in use, but none of these can be relied upon always to give good results. A useful method is as follows:

Boil some clean cover glasses for ten minutes in 5 per cent. caustic soda solution; allow to cool; wash with distilled water; and then boil for ten minutes in 10 per cent. hydrochloric acid. Again wash with distilled water and finally with a mixture of ether and alcohol. The forceps used for lifting them must be thoroughly clean.

Take an agar culture of some bacteria, known to be actively motile, which has been kept for twelve to twenty-four hours at 38° C. (§ 29). To a drop of tap water on an object slide add a loopful of the water of condensation from the agar culture. The water and the object slide should be previously warmed in the incubator. Allow the bacteria to spread without assistance throughout the water, and any conglomerates to settle. After a few minutes transfer a loopful of this water to one of the specially prepared cover glasses; the drop should spread at once over the glass. Put the cover glasses into the incubator as soon as prepared, or the latter part of the work may be done with advantage entirely inside the incubator. When dry, apply a mordant, *e.g.* the preparation from Bunge (25 c.c. of a 25 per cent. solution of ferric chloride is mixed with 75 c.c. of a concentrated solution of tannin in water; after standing for several days, or weeks, the solution is ready for use). Filter the mordant through a small filter paper on to the dried specimen, and allow it to act under gentle warming for one to two minutes. Then wash the preparation thoroughly and dry it. Next stain for one to two minutes with warm carbol fuchsin (1 part conc. alcoh. fuchsin + 9 parts of a 5 per cent. aqueous carbolic acid solution). Finally wash, dry, and mount the specimen in Canada balsam in the usual way. In order to get very clean specimens the preparation may be washed before applying the mordant, and after staining, with a weak solution of acetic acid (*see* § 37).

References: The corresponding chapters in the books on the microscope and general bacteriological technique quoted on pp. 1 and 2; *also* Lehmann and Neumann's *Grundriss der Bakteriologie.*

VI. EXAMINATION OF CULTURES.

28. **Plate Cultures.** The isolating of bacteria was first accomplished by R. Koch and his pupils by pouring the infected gelatine on to glass plates. The cultures are now

almost always made in "Petri dishes," but they are still conventionally called "plate cultures." Gelatine (§ 20) or agar (§ 34) media serve for this purpose. The appearance of the colonies has already been described in §§ 18 and 21. The growth and development of the pure cultures of the air and water bacteria should be carefully observed.

FIG. 23. Soyka Flask, with "monster" colony of *Azotobacter*, about ⅔ natural size.

For exact experiments, instead of the ordinary Petri dish, the "**Soyka Flask**" may be used, which makes foreign infection almost impossible. The medium is sterilised in the same way as in test tubes, and the "plates" are obtained by simply laying the flasks on their sides.

Further, by inoculating the centre of the medium with a small quantity of the bacterial mass, "monster" colonies can be obtained which give very characteristic appearances (Fig. 23).

29. **Streak Cultures.** To make a streak culture, dip the platinum loop into the bouillon culture and draw it straight over the entire length of the solid agar or potato slope, beginning, in the case of the agar, at the water of condensation. Avoid scratching or breaking the surface of the agar. In the case of the potato culture it is better to rub the potato surface, and not merely make a simple streak. On agar, especially when a freshly isolated culture is kept at 38° C., a rapid development usually takes place; a well-spread growth is generally apparent in one day. Place the cultures in the test-tube stand, and examine them daily as to colour, brightness, consistency, smell, nature of

the surface and edge, odour, &c. Crystals of various kinds ($MgNH_4PO_4$, etc.) are often to be found in old agar cultures.

As gelatine is liquefied by many bacteria it is seldom used for streak cultures. On the other hand, very few bacteria are known which can liquefy agar (*cf.* Gran, *Centralbl. f. Bakt.*, II. Abt. 9, p. 562, and Biernacki, *ibid.*, 29, p. 166).

30. Stab Cultures. Remove a small quantity of the material from an agar streak culture with the platinum needle and insert this into a tube with gelatine or glucose agar (Fig. 24). The stab should be exactly down the centre of the medium in the tube and should reach to the bottom. When making the stab be careful to avoid splitting or breaking up the medium. Gelatine which has become hard and therefore splits readily should be remelted and allowed to solidify before being used for this purpose. Glucose-agar stab cultures are used mainly as a test for gas production; and the nature of the surface growth, the development along the stab (extent, strength, appearance), as also the nature of any liquefaction should be carefully watched and noted in the gelatine cultures (Fig. 25).

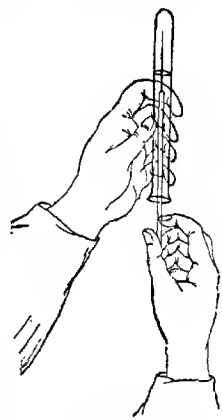


FIG. 24. Making stab culture.

31. Liquid Cultures. With the platinum loop inoculate a tube of milk from the bouillon culture. The milk may remain unchanged or various effects may be produced. It may be coloured, or coagulated owing to formation of acid or rennet, or peptonised, or an abnormal alteration of taste, smell, or consistency (production of slime or gas) may be

observed. The bouillon cultures should be examined as to the intensity and kind of turbidity, for skin or ring development on the surface, formation of precipitate, colouration, smell, alteration of consistency, &c.

The test for **indol** is important from a diagnostic point of view. The method most commonly used (Salkowski's) is as follows: A

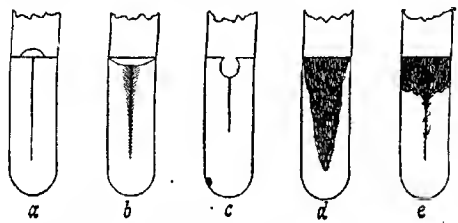


FIG. 25. Gelatine stab-cultures. ½ natural size.

(*a*) Gelatine not liquefied, so-called pin-head culture; (*b*) concave liquefaction, root-like projections from stab (villous); (*c*) spherical liquefaction (napiform), little development in stab; (*d*) conical liquefaction; (*e*) cylindrical liquefaction.

bouillon culture, at least one week old, is first warmed with about 4 c.c. of 10 per cent. sulphuric acid, and then with ½–2 c.c. ½ pro mill. sodium nitrite. A pink colouration indicates indol.

A more reliable method is that of **Ehrlich**: 5 c.c. of a hydrochloric acid solution of dimethyl-amido-benzaldehyde (4 grms. para-dimethyl-amido-benzaldehyde + 380 c.c. 96 per cent. alcohol + 80 c.c. concentrated hydrochloric acid) is first added to the bouillon culture; then 5 c.c. of a saturated aqueous solution of potassium persulphate; an intense red colour indicates indol. These solutions can be obtained ready for use from Grübler and Co. in Leipzig. (*Cf.* A. Böhme, *Centralbl. f. Bakt.*, I, Abt., Orig. **40**, p. 129.)

The evolution of sulphuretted hydrogen can be tested by holding a strip of filter paper moistened with lead acetate inside the tube (*see also* § 132).

Cultures must in every case be distinctly and accurately labelled, giving the journal number, medium, and date.

32. **Testing various sources of Carbon and Nitrogen.** In most cases quite sufficient information for the identification of any kind of bacteria is obtained by cultivating on the six standard media. Sometimes, however, it is desirable to test whether other sources of carbon and nitrogen are utilised. The special requirements of some of the organisms found in dung and soil will be dealt with later (Parts C and D). At present only experiments for general diagnoses will be dealt with.

FIG. 26. Production of gas in sugar-gelatine shake culture.

Besides **glucose**, other sources of carbon may be considered, *e.g.* **other sugars (lactose, cane sugar, maltose, galactose,** etc.); **alcohol, mannite, glycerine,** etc.; **fats; salts of organic acids (lactates, malates, succinates,** &c.). These substances are usually added in the proportion of ½-2 per cent. to sugar-free bouillon.

Bouillon is freed from sugar by infecting the culture with lactic acid streptococci or coli bacteria and incubating for one or two days at 30°–38° C. The broth is then heated, neutralised, filtered, and sterilised again.

Acid formation is tested by the addition of litmus.

Shake cultures in dextrose- or lactose-gelatine often give very instructive results. They are prepared by inoculating the liquid gelatine, mixing thoroughly, and then causing the gelatine to solidify quickly. If the sugar is decomposed many bubbles of gas are to be seen in the gelatine (Fig. 26).

Th. Smith's fermentation tube (Fig. 27) is generally used for collecting the gas evolved from fermenting liquids. The gas collects in part in the long arm of the apparatus, where it can be measured by means of the special graduations on the tube. It can also be tested qualitatively for CO_2 and H. The apparatus is somewhat difficult to clean and is easily broken. Another method, Burri and Düggeli's

(*Centralbl. f. Bakt.*, I. Abt., Orig. **49**, p. 155 *et seq.*), is as follows: 10 c.c. of the infected sugar-agar is filled into a thick-walled glass tube 40–50 cm. in length. After this has solidified a further layer of sterile warm water-agar at 60°–80° C. is poured on. The glass tube is kept in a horizontal position in the incubator; the movement of the "sliding agar plug" shows the intensity of the gas development. More complicated apparatus for exact gas analyses have been constructed by Epstein (*Centralbl. f. Bakt.*, II. Abt., **6**, p. 658), by Hofstädter (*ibid.*, **13**, p. 765), by Beijerinck and Minkman (*ibid.*, **25**, p. 32). It is generally sufficient to close the tube or flask, which has been completely filled, with a perforated rubber stopper and collect the fermentation gases over water by means of a properly bent glass tube. The rubber stopper with the glass tube should be sterilised in the autoclave.

FIG. 27. Fermentation tube (Th. Smith), ½ natural size.

The following solutions, filled into test tubes in the usual way and sterilised on three successive days, serve to test which sources of *nitrogen* can be used in particular cases.

Peptone water: 100 tap water, 1 peptone (Witte), 1 common salt; filtered after heating.

Cohn's solution: 100 distilled water, 1 amm. tartrate, 0·5 K_2HPO_4, 0·5 $MgSO_4(+ 7H_2O)$, 0·05 $Ca_3(PO_4)_2$; not filtered.

Uschinsky's solution: 100 dist. water, 3–4 glycerin, 0·6–0·7 amm. lactate, 0·3–0·4 sodium aspartate, 0·5–0·7 NaCl, 0·2–0·25 K_2HPO_4, 0·02–0·04 $MgSO_4$, 0·01 $CaCl_2$.

Fränkel's solution: 100 dist. water, 0·6 amm. lactate, 0·4 asparagin, 0·5 NaCl, 0·2 K_2HPO_4; made distinctly alkaline with dilute NaOH.

Maassen's solution: 100 dist. water, 0·7 citric acid, neutralised with pure KOH; then 1 asparagin, 0·2 K_2HPO_4, 0·25 crystallised Na_2CO_3, 0·04 $MgSO_4$, 0·001 $CaCl_2$; 1·5–4 per cent sugar, glycerine, mannite, etc., is added as may be desired.

See also the culture solutions given by A. Meyer (*cf.* Gottheil, *Centralbl. f. Bakt.*, II. Abt., **7**, p. 432, and A. Meyer, *Praktikum der botan. Bakterienkunde*, 1903, p. 22 *et seq.*); also the special media given in Parts B–D.

VII. HAY BACTERIA. SPORES OF BACTERIA.

33. **Cultivation of Hay Bacteria.** Add several grammes of finely cut hay to tap water in a beaker and stir thoroughly. After one to two hours filter the mixture through cloth, pour about 100 c.c. of the filtrate into a 300 c.c. Erlenmeyer flask, and heat for ½ hour in the steam steriliser. Put other 10 c.c. of the filtrate into a 50 c.c. Erlenmeyer flask, and place both flasks in the incubator (38° C.). As soon as growth begins make Chinese ink and hanging drop microscopic preparations. In the liquid which has not been heated all kinds of bacteria, moulds, and protozoa will be found, but in the pasteurised solution only large, motile bacilli will be visible, possibly with spores, showing as round, highly refractive cells (*see* Plate I., Fig. 4). Prepare gelatine and agar plates from the pasteurised liquid and describe in detail the various stages of growth of the resulting colonies. The hay bacillus (*Bac. subtilis*) is not always obtained, but sometimes related forms, more often the so-called potato bacillus (*Bac. mesentericus*). Isolate one or two of the spore-forming species and cultivate them on the standard media.

34. **Preparation of Agar Plates.** Agar plates are commonly used instead of gelatine for those bacteria—often spore-bearing—which thrive better at 38° C. than at 20° C. It should be remembered that agar does not become liquid under 100° C. and solidifies at 40° C. Melt the required number of agar tubes in a beaker of water (in steamer or over the flame). Reduce the temperature of the water to 45° C. by adding cold water and keep it at this temperature by means of a small flame; about five to ten minutes later the temperature of the agar will also have gone down to 45° C. Inoculations can now be made in the usual way (§ 21) without the bacteria being injured by too high a temperature. The work must, however, be done *expeditiously*, otherwise the agar may become prematurely solid. Agar tubes not being used at the moment should

always be returned immediately to the water bath. The mistaking of one tube for another can be avoided by making some simple mark on the cotton-wool plug, such as twisting out one or two portions into points.

After pouring each tube, move the plate about quickly so as to spread the agar thoroughly. After the agar has become solid, turn the dishes upside down. Place a piece of filter paper (about 4 cm. sq.), which has first been passed through the flame, inside the lid of the Petri dish, and moisten the paper with a drop of glycerine. Place the plates in the incubator, still upside down to prevent the water exuded from the agar spreading over the surface of the medium. The glycerine tends to draw the water from the surface of the medium. Observing these precautions prevents the colonies on the agar growing through each other, as otherwise often happens.

35. **Gram's Method of Staining.** This method has a special value for diagnostic purposes. The process is as follows:

1. *Spread* and *fix* on a slide in the usual way a little of a one to two days old bouillon or agar culture of the hay bacillus.

2. *Stain*, warming gently, for two minutes with *aniline Victoria blue* of the following composition:

3-4 c.c. conc. alc. Victoria blue, 5 drops aniline oil, 15 c.c. absol. alcohol, 30 c.c. dist. water,	Ready for use twelve to twenty-four hours after being mixed.

3. Pour off the stain, and replace with a solution of *iodine potassium iodide* (Lugol's solution: 100 dist. water, 1 KI., 0.3 I.) for two minutes.

4. Pour off the solution, and wash with *absolute alcohol* till no more colour is removed.

5. Finally *wash* with water, *dry and mount* in Canada balsam in the usual way.

Gram's method is a test of decoloration. Many bacteria give up their colour on being treated in the above

way with alcohol. Others, *e.g.* the hay bacillus, do not. When taken from old cultures, bacteria which are really "Gram positive" may appear to be "Gram negative"; and many organisms, as, for example, the leguminose nodule bacteria, react with even greater irregularity.

A streak culture of the hay bacillus may be conveniently used for control purposes. When other bacteria are being tested and some doubt arises as to whether they are + or −, they may be mixed with "hay" bacteria from a young culture and the relative intensity of the stain in the two sorts compared.

36. **Spores of Bacteria.** In cultures of the hay bacillus eight to fourteen days old (especially from hay extract, agar, or potato slopes) there is usually an abundance of bacilli containing spores, also of free spores. Rapid spore-formation can usually be brought about by inoculating from an actively growing culture (bouillon, agar, &c.) into sterile water.

In the hanging drop the spores already formed are usually easily recognised as highly refractive bodies, especially when the diaphragm of the microscope is fairly well closed down. In the ordinary stained preparations growing spores (*i.e.* concentrated albuminoid) are more intensely stained than the rest of the bacteria. Mature spores, on the other hand, remain unstained in their interior, only their outer layer being slightly coloured. When still in the parent cell they are seen as clear round spots within the stained cells.

It is not always possible to decide by these direct observations whether spores are present or not, as vacuoles, drops of oil, &c., can be mistaken for spores. In order to make quite certain it is often necessary to pasteurise the medium, to stain the spores, or to observe the spores germinating.

Pasteurising. Inoculate bouillon with two or three loopfuls from a culture presumably spore-bearing. Heat to 80° C. for an hour; and observe whether further growth takes place.

Put the bouillon tube and an open tube with about 10 c.c. of water into a water bath. Place a thermometer in the open tube and heat the water, keeping the temperature at about 80° C.

To get more exact information regarding the heat-resisting powers of the spores, place a large number of bouillon tubes, inoculated with spore-bearing bacteria, in a water bath at 100° C. Take out a tube at regular intervals (say every two or five minutes). and immediately cool it in cold water.

Staining of Spores. Many methods are available, of which the following is one :

1. Make a fixed *preparation* on the *slide* in the usual way.
2. Cover with *carbol fuchsin* (1 part conc. fuchsin and 9 parts of a 5 per cent. solution of carbolic acid in water).
3. *Warm* the specimen over a small flame till steaming commences ; put aside for a little, and then warm again. Repeat three or five times, warming altogether for three to five minutes.
4. Pour off the stain and wash with 3 per cent. *hydrochloric acid-alcohol* for half to one minute till the red colour has almost gone.
5. *Wash* thoroughly with water, *restain* with an aqueous solution of methylene blue (20–30 sec.), *dry and mount* in Canada balsam.

The spores should appear red and the bacilli blue. It may be found necessary to lengthen the time of staining with warm carbol fuchsin or to remove the colour more thoroughly with hydrochloric acid-alcohol. Spores which are very difficult to stain may be treated previously for half to two minutes with a 5 per cent. chromic acid solution.

Spore germination. The best and only positive proof of the presence of spores is the direct observation of their germination. This is often a tedious process, but only requires to be done in exceptional cases.

From a culture rich in spores take sufficient material and mix it with a little water in a test tube, until a thick turbidity is obtained. Heat the tube to 80° C. for five to ten minutes. Spread two or three loopfuls of the pasteurised material on a cover glass previously

sterilised in the flame, and put a drop of liquid agar on the smear. When solid proceed as for the "hanging drop" (§ 25). Sterilise the glass slide in the flame also. In this case Canada balsam must be used, as the melting point of vaseline is lower *than 38° C. Put* the specimen in the incubator and examine it from time to time (every half to one hour). As soon as spore germination begins the process can be observed under the microscope at room temperature. Special contrivances for this purpose are expensive, but are not indispensable.

VIII. DESCRIBING AND IDENTIFYING BACTERIA.

37. **Describing Bacteria.** The student should become accustomed from the first to write out an exact and complete account of what he has observed. Simple sketches are often very useful. Two pages from a laboratory notebook are reproduced on pp. 50 and 51, and illustrate a method which has frequently proved useful. At the top of the left page are written the provisional letters denoting the particular culture, and on the top of the right hand page the technical name of the organism as soon as this is confirmed by the experimental evidence. The examination of the cultures will generally occupy not less than four weeks, and longer if fuller knowledge is required.

The shape and size of the bacteria should be determined from material out of different media, preferably from agar, bouillon and potato.

A very small quantity is taken from the solid media, on the platinum needle, and mixed carefully with a drop of water. In order to secure quite clean specimens, the preparation may be treated for a moment, before washing with water, with very dilute acetic acid (1 acetic acid: 200 water), the acid washed off and the preparation quickly dried. By this treatment solid particles of media are removed.

From milk cultures very good specimens can often be obtained in Chinese ink (*see* § 24). If the specimen is to be stained, the fat must first be removed with alcohol and ether; in this case the bacteria are fixed by the treatment with alcohol. Special methods of preparing well-stained slides from curdled milk are described in § 60.

W. 2 (green).

Form and Size: Rods, rounded at the ends. Living, $\frac{1}{2}$-$\frac{3}{4}$ μ thick, $1\frac{1}{2}$ μ long; often in twos, also long threads. Stained, rather thinner. Threads, 10–15 μ long.

Motility: Active.

A

Staining: Good with ordinary aniline stains. Gram negative.

Sporulation: Not observed.

Flesh Gelatine Plate: **Surface colonies — macroscopic:** Round, opaque film; concave liquefaction. Liquefied gelatine turbid. Colonies become crumbly. Gelatine coloured green.

B

Surface colonies — microscopic (× 50): Young colonies round, of irregular structure, with hairy edge (Sketch A). Older colonies compact in centre, becoming crumbly towards outside (Sketch B).

Deep colonies — macroscopic: Mostly whitish-yellow small points and discs, partly bluish-white, irregularly edged films.

Deep colonies—microscopic: Point-like, yellowish-brown colonies, with tuberous, irregular, notched edges. Filmy, strongly granulated colonies, light-grey, with irregular, torn edge.

Bact. fluorescens (Flügge) Lehm. and Neum.

Flesh Agar Streak: Shallow, opaque, grey-white, glistening growth along the streak. Edge finely indented. Round the streak a thinner, grey, and more transparent layer on the agar. Water of condensation clear, with whitish precipitate. Agar with pronounced green fluorescence. Unpleasant smell.

Glucose Agar Stab: Fairly good, thread-like growth in stab. Grey-white, shining, spreading, surface growth. Agar fluorescent. No gas.

Flesh Gelatine Stab: Good development in stab, yellowish. Grey-white surface growth with finely folded edge, concave depression. Liquefaction later cylindrical. Yellow-green fluorescence.

Bouillon: Very turbid. Incoherent film on the surface. Green fluorescence. Unpleasant smell. Distinctly alkaline. No indol.

Milk: Slowly clarified (in layers). Coloured green from surface downwards.

Potato: Yellowish, greyish-brown, dull, shining growth.

Remarks: Glucose-bouillon: no gas, alkaline reaction.
Saccharose-bouillon and lactose-bouillon: the same.
Nitrate bouillon: nitrate disappears without gas production.

If an organism is being examined which does not appear to be described in the reference books, the experiments should be repeated several times, and prolonged as much as possible, in order that the limits of variation, which are in many cases very considerable, may be defined.

The number of quite insufficiently described organisms is extraordinarily large. A further increase of such "new" species, as a result of hasty research, is to be earnestly deprecated.

If no description can be found in the books of reference which entirely agrees with the results of the investigations, it will generally be sufficient to state, in few words, in what respects the form under consideration differs from the one resembling it most closely.

A very serviceable plan is to record the characteristics by means of the following notation of the Society of American Bacteriologists:

100	Sporing.
200	Non-sporing.
10	Obligate aerobic.
20	Facultative anaerobic.
30	Obligate anaerobic.
1	Gelatine liquefied.
2	Gelatine not liquefied.
·1	Acid and gas from glucose.
·2	Acid, but no gas from glucose.
·3	No acid from glucose.
·4	No growth with glucose.
·01	Acid and gas from lactose.
·02	Acid, but no gas, from lactose.
·03	No acid from lactose.
·04	No growth with lactose.
·001	Acid and gas from saccharose.
·002	Acid, but no gas, from saccharose.
·003	No acid from saccharose.
·004	No growth with saccharose.
·0001	Denitrification, with evolution of gas.
·0002	Nitrate not reduced.
·0003	Nitrate reduced with out gas production.
·00001	Fluorescent.
·00002	Violet colouration.
·00003	Blue colouration.
·00004	Green colouration.
·00005	Yellow colouration.
·00006	Orange colouration.
·00007	Red colouration.
·00008	Brown colouration.
·00009	Pink colouration.
·00000	No colouration.

e.g. the numbers for *Bact. fluorescens* are: 221·33331, *Streptoc. lactis*: 222·22220.

38. **Identification of Bacteria.** The identification of an isolated bacterium is not always easy. The tables given in Appendix I. will serve for preliminary purposes, but for more exact identification reference must be made to the works on General Bacteriology and the special Agricultural Bacteriological Diagnoses mentioned on p. 2.

Compilations professing to give full particulars of the different species of bacteria, such as those by Migula and Matzuschita, are often most inaccurate, and are practically useless for the beginner. The *Atlas und Grundriss* by Lehmann and Neumann is for almost all cases very useful. Though in this work pathogenic germs receive the most attention, still the Agricultural Bacteriologist is seldom left disappointed. The arrangement of the Identification Tables in Appendix I. is similar to that in Lehmann and Neumann's book, with certain abbreviations and additions necessary for our special purpose.

The descriptions of certain bacteria of special importance in agriculture not found in Lehmann and Neumann must be sought for in the original works, which are quoted in the author's *Handbuch der landwirtschaftlichen Bakteriologie*. (*Compare* also the special notes in the different chapters in Parts B–D.)

39. **Further Cultivation of Pure Cultures.** Pure cultures can only be kept typical and active with any certainty by transferring them to fresh media regularly and frequently. Spore-forming bacteria will often lose this power if the cultures are not pasteurised from time to time. The isolated species can be best kept for later experiment on an agar slope.

Cultures of new species should be given to the Bacteriological Museum and Bureau for the Exchange of Bacterial Cultures at the American Museum of Natural History, New York, or to Král's bakteriologisches Museum (Prof. Kraus and Dr. Pribram), Wien IX/3 Zimmermannsgasse 3, where material for comparison can be found.

IX. POTATO BACTERIA. ANAEROBIC METHODS.

40. **Cultivation of Potato Bacteria.** Pierce an unwashed potato with a knife in several places, and put it in a beaker with water. Place the beaker, covered with a glass dish, in the incubator at 38° C. After a day or two examine by the hanging drop and by Gram's staining methods. These should give very interesting and varied pictures. Under aerobic conditions various forms of the potato bacillus group (*Bac. mesentericus*) are to be seen, while under anaerobic conditions the butyric bacilli (*Bac. amylobacter*) are most abundant. The latter, when sporulating, often assume a characteristic, swollen, club-shaped form, the so-called clostridium.

41. **Isolating Obligate Anaerobes.** Clean two Burri tubes thoroughly. Close one end of each with a rubber stopper and the other with a cotton-wool plug. Sterilise them in the autoclave (2 atms.) or in the steam steriliser (two hours). Melt three flesh-agar and three glucose-agar tubes, and cool to 45° C. Inoculate from the potato solution, making the first inoculation with the platinum needle and the next with the loop, in the way already described. Now hold the Burri tube upright, with the rubber stopper on the bench, and pour in the third, *i.e.* the weakest dilution. Cause to solidify quickly by placing the Burri tube in cold water. Next pour in the second dilution, and when this is solid, the first dilution on the top. Incubate both Burri tubes at 38° C. In one or two days the results illustrated in Fig. 28 should be obtained, *i.e.* very considerable gas development in the glucose-agar and little or none in the other. Distinct colonies, as a rule, are found only in the latter.

The further investigation of the colonies may be carried out as follows. Place a sheet of filter paper on a porcelain plate. Select some well-isolated colonies in the tube. Remove the rubber stopper, and, holding the tube in a sloping position, allow the agar to slide out of the tube so as to fall

on the filter paper. Sterilise the blade of an old pocket knife in the flame and cut sections of that part of the agar in which the selected colonies are enclosed. Sterilise the knife after cutting each section. Place each section on a slide and examine it microscopically in the same way as with plate colonies. Infection from the air can be prevented by covering with sterilised cover glasses. When these examinations have been made, inoculate from a well-isolated colony a bouillon tube and keep this at 38° C. under anaerobic conditions (*see* § 42). When sufficient growth is apparent purify by cultivating again on flesh-agar in Burri tubes, or in Petri dishes (*see* § 43).

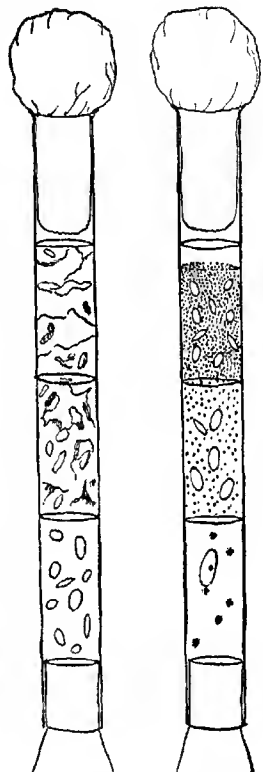


FIG. 28. Isolating Anaerobes in Burri tubes (¾ natural size). Right, flesh-agar. Left, glucose-agar.

42. **Cultivation of Obligate Anaerobes in Test Tubes.** The method is almost the same as for aerobic cultivation, only *thick-walled* glass tubes are used. Thoroughly clean the tubes, fill them with the proper medium, close with cotton-wool plugs, and sterilise once or twice. In this case

use non-absorbent cotton-wool instead of the ordinary absorbent wool. The plugs should be made fairly tight, and should not project far out of the tubes. Prepare the following solutions and hold them in readiness: 25 c.c. of a 20 per cent. solution of pyrogallic acid, and 25 c.c. of a 20 per cent. solution of caustic potash.

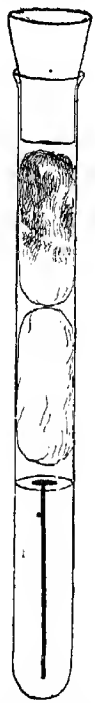


Fig. 29. Anaerobic culture in tube closed after the method of Wright-Burri (⅔ natural size).

Anaerobic conditions are secured in the tubes by the method of Wright-Burri (Fig. 29) in the following way: After inoculating the medium in the tube push the plug, by means of a glass rod, into the tube till it is about 1 cm. above the surface of the medium. Over this, place a rather loose plug (not sterile) of absorbent wool so that the top is about 2 cm. below the mouth of the tube. On this plug pour first 2 c.c. of the pyrogallic acid solution and then 2 c.c. of the potash solution. Close the tube *immediately* with a close-fitting rubber stopper, and, if necessary, make it quite air-tight with paraffin.

If the agar slope is used it should be made rather shorter than usual. This very convenient method makes the large number of complicated methods of anaerobic cultivation quite superfluous. When making inoculations from this tube, first remove the rubber stopper, then, by means of strong forceps, the cotton-wool with pyrogallic acid, and finally bring the sterile cotton-wool plug to its usual position. If care is taken the culture ought not to be soiled by the pyrogallic acid.

43. **Plating Anaerobic Bacteria.** The following method given by Heim is very useful. Make plate cultures in the usual way and place a ring of plasticine, corresponding in size to the circumference of the lower part of the Petri dish, on a circular glass plate of 13 cm. in diameter. Within this ring place a piece of cotton wool (about 0.5 grm.) and moisten it with 1 c.c. of the pyrogallic solution. Hold the under half of the Petri dish containing the culture upside down above the wool, on to which pour 1 c.c. of the potash solution. Press the dish quickly into the plasticine smear so as to make it air-tight. In this way strictly anaerobic conditions in the Petri dish are obtained quite simply. If the work is well done, the wet cotton wool is firmly fixed on the glass plate, and it is possible to turn the plate to its original position, which is desirable, especially with gelatine plates containing liquefying bacteria.

Another method is that of Burri. Special, small, rectangular glass troughs are sterilised in Petri dishes, and into these the infected gelatine is poured. When the medium has solidified the hands are sterilised by washing with soap, and dipping for some minutes into 1 in 1000 corrosive sublimate, and the troughs put into wide tubes which are closed as stated in § 42 (*see* Fig. 30).

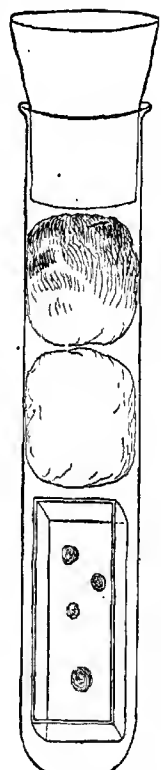


FIG. 30. Anaerobic plate culture, *after Burri* (⅔ natural size).

When growth has become apparent, the small troughs are placed again in sterile Petri dishes preparatory to further examination.

References: Heim, *Centralbl. f. Bakt.*, I. Abt., Orig., **55**, p. 337; Wright, *Centralbl. f. Bakt.*, I. Abt., **29**, p. 61; Burri, *Centralbl. f. Bakt.* II. Abt., **8**, p. 533; Kürsteiner, *ibid.*, **19**, pp. 1, 97, 202, 385, spec. pp. 21 *et seq.* and 97 *et seq.* For other methods see the respective chapters in the text books on general bacteriological technique.

X. SPECIAL METHODS.[1]

44. **Auxanography.** The auxanographical methods of Beijerinck may be used to ascertain the necessary, or most suitable, food materials for different organisms. Gelatine or agar, prepared with water, or pure mineral solutions, is inoculated strongly with the particular germs and poured into Petri dishes. When the medium has solidified (with agar, after thorough drying), sterile drops of the different solutions to be tested are placed upon it. The substances diffuse into the medium in different ways. Wherever a favourable combination takes place there the germs grow. From the resulting "auxanogram" conclusions may be drawn.

Reference: Beijerinck, *Centralbl. f. Bakt.*, **7**, 1890, p. 347.

45. **Test of Symbioses or Antagonism.** It is often desirable to know whether two or more different species growing together help or hinder each other. This can be tested by a method similar to the auxanographical methods. Inoculate a particular medium with one species of germ, and, after the plate has become solid, make parallel or cross streaks on the medium with other bacteria. At the same time inoculate solutions of different composition with approximately the same quantity of the different species, and observe whether the combination proves favourable or unfavourable.

46. **Enriching[2] Methods.** The enriching methods used with great success by Prof. Beijerinck in Delft are based on the same prin-

[1] The methods which are dealt with in §§ 44–48 are not only of general interest, but are of importance in reference to certain investigations in the bacteriology of milk, dung, and soil.

[2] Increasing the number of one type of germ relatively to other types.

ciple. Amongst a complex mixture of bacteria, the different species which take part in any one particular change can be ascertained and increased by determining and regulating the chemical and physical conditions (air, temperature, &c.), in a suitable manner. These methods are of special importance for the bacteriological examination of dung and soil (*see* Parts C and D). Spontaneous fermentations, as in the making of silage, souring of milk, ripening of cheese, etc., and diseases of bacterial origin are often due to a spontaneous relative increase of, or enriching in, the specific organisms. In the laboratory the influence of chance can be to a large extent eliminated. In the most successful experiments of this sort only one species remains, and, what is of great importance, all possible varieties of this species may be seen together, varieties which might readily be taken for quite different species if examined by the usual methods of investigation and isolation. As a matter of fact, many of the so-called different species of bacteria are undoubtedly merely different varieties of the same germ insufficiently studied. Mistakes of this kind can be easily avoided by adopting enriching methods.

47. **Dilution Methods.** The solutions used for the above enriching methods will serve also for numbering the bacteria of the different physiological groups in dung, soil, &c. Many of the more important bacteria in agriculture do not grow at all or only very slowly on the usual media. Solid media are in many cases particularly unsuited for their development. Culture solutions are made, inoculated with a little of the original material, and various dilutions made from it in the usual way. At least four tubes, as controls, must be infected from each dilution. The same quantity of earth, dung, milk, etc., which is added to the first series of tubes must be added also to the other tubes to make the chemical and physical conditions exactly correspond. Of course, all the media in this case must be sterilised before inoculating. The approximate number of bacteria causing any particular change (*e.g.* forming nitrate) in 1 grm. of the material may be found by calculation.

The results cannot be quite accurate since it must be assumed that in the last dilution in which growth is found only one germ was present.

48. **One-Cell Culture.** Though the plating method—of R. Koch—gave a much greater degree of probability than the older

dilution methods that the colonies obtained really originated from one germ, still, absolute certainty was wanting. This can be secured only by the one-cell method, which E. Chr. Hansen has for a long time used in his investigations of the comparatively large yeasts. A similar method for the cultivation of bacteria was not available until lately. The *point culture with Chinese ink*, introduced by R. Burri, solves this problem. With a large platinum loop, place three or four separate drops of the properly diluted, sterile emulsion of Chinese ink on a clean, sterile slide, and make dilutions in these drops from the germ-containing material. For the first dilution use the platinum needle and for subsequent ones, the loop. By means of a drawing-pen, previously sterilised in the flame, put the smallest possible droplets—about ·1 mm. in diameter—from the weakest dilution on to a sterile gelatine plate, one of a number previously prepared. The pen should be brought in a horizontal position near the gelatine, but not allowed to touch the medium. Cover the row of droplets on the gelatine with small, sterile, cover-glasses (about 3 × 3 mm.) and examine under the high power. A drop which contains only one germ is either left where it is and the germ allowed to develop a colony there, or, by means of sterile forceps, is removed to a special medium. The germ adheres to the dry cover-glass rather than to the wet gelatine.

Reference: Burri, *Das Tuscheverfahren*, 1909; *Centralbl. f. Bakt.*, II. Abt., **20**, p. 95.

B. DAIRY BACTERIOLOGY.

XI. NUMBER OF BACTERIA IN MILK. SEDIMENT TEST.

49. **Media.** Two hundred and fifty test-tubes are prepared, and two litres of the cleanest fresh skim milk and also three or four potatoes procured. The following media are prepared according to the directions given in §§ 6–13:

30 tubes of flesh gelatine.	15 tubes bouillon.
30 tubes of flesh agar.	30 tubes milk.
15 tubes of glucose agar.	15 potato slopes.

Milk sugar (·5 per cent.) is added to 120 c.c. of the remaining bouillon, and to the rest .05 per cent. æsculin and .05 per cent. ferric citrate. This gives fifteen tubes of *lactose bouillon* and about ten tubes of *æsculin bouillon* for the coli test, § 58.

The æsculin bouillon should not be sterilised in the autoclave, especially if Ragit is used in the preparation.

Whey Gelatine and *Whey Agar* are prepared in the following way: The $1\frac{3}{4}$ litre of skim milk which still remains is warmed to 35° C. and curdled with rennet. The coagulum is broken up by stirring with a glass rod and the mixture heated on the water bath to 80° C. It is next poured through cloth, and 1 per cent. Witte's peptone and $\frac{1}{2}$ per cent. sodium chloride (or common salt) added to the filtrate, which is then heated in the steriliser for an hour and finally filtered through filter paper. Two hundred and fifty cubic centimetres of the filtrate is utilised for the preparation of whey gelatine and whey agar respectively, in the same way as with bouillon, the remainder being sterilised in a 2-litre

flask and kept for later use. Whey media should not be heated in the autoclave, or, if so, only to ½ atm.

The above supply of media should be about sufficient for the exercises given in Ch. XI–XVIII, provided more detailed investigations are not undertaken.

Other media may also be required as: *Heyden agar* (100 dist. water, 1 "Nährstoff Heyden," 1 agar); *Flesh-peptone-whey-gelatine* (containing flesh bouillon and whey in equal parts), *lactose gelatine* or *lactose agar* (flesh gelatine or agar + 2 per cent. lactose). To the latter *litmus* may be added to demonstrate acid formation, or the acidity may be ascertained by adding chalk (*see* § 60).

50. Germ Content of Milk. For the preparation of the necessary dilutions (*see* § 20) sterilise a 300 c.c. Erlenmeyer flask containing 99 c.c. water, ten test tubes with 9 c.c. water each, ten 1 c.c. pipettes, and twenty-four Petri dishes. Owing to the variations in the bacterial flora in different samples of milk, the best results are sometimes obtained from one medium and sometimes from another. Hence six tubes of each of the following media should be held in readiness: flesh gelatine, flesh agar, whey gelatine and whey agar. From those milk samples which presumably contain comparatively few bacteria three dilutions should be made, so that the respective Petri dishes represent dilutions containing $\frac{1}{10}$, $\frac{1}{100}$, $\frac{1}{1000}$ c.c. of milk, while from ordinary market milk 1 c.c. should be added to the 99 c.c. water in the flask and from this dilutions corresponding to $\frac{1}{10.000}$, $\frac{1}{100.000}$, $\frac{1}{1.000.000}$ c.c. made. Since the germs in milk often stick together in clumps the various dilutions should always be vigorously shaken.

Gelatine plates are kept at 20° C. for eight to ten days, agar plates (upside down with glycerine paper on the lid) for three days at 38° C. (*see* §§ 17 and 34). Then the colonies are counted and examined carefully. The most frequently occurring types should be inoculated into fresh medium, replated (§ 21) to get pure cultures, and examined in the usual way (*see* Ch. V–VIII).

The counting of the colonies can be quickly accomplished by dividing the work between two or four students. When possible the tests given in §§ 51–56 should be started at the same time as the above, from the same sample of milk.

The above dilutions can also be used for the coli test (§ 58). Of course, for exact work duplicate experiments are indispensable.

51. Sediment Test. In many cases the laborious and not too accurate plate methods may be replaced by

FIG. 31. Sedimentation tubes, *after Trommsdorff* ($\frac{1}{2}$ natural size).

FIG. 32. Centrifuge ($\frac{1}{8}$ natural size).

direct microscopic examination of the sediment obtained by centrifuging. This test is often specially useful in examining faulty milk. Ten cubic centimetres of the well-mixed milk, which has been warmed to 60°–70° C., is filled into a special tube having a narrower part at the lower end (Fig. 31) and centrifuged in a machine for this purpose (Fig. 32) for about five minutes, till a more or less considerable, compact, sediment settles out. This remains in the narrow bore when the milk is poured off. If necessary, the centrifuging can be repeated. Dirty milk yields a

grey to brown sediment, sour milk a white precipitate (casein). Sometimes also a quantity of cream-coloured sediment is separated out, especially when the milk is obtained from cows with **gargety udders.**

When the milk has been poured off, a stained microscopic preparation is made from a small quantity of the sediment. An aqueous solution of methylene blue is the best stain for this purpose. Illustrations of characteristic

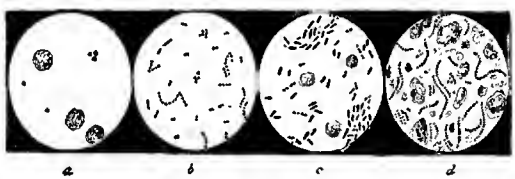


FIG. 33. Microscopic appearances of residues from Sediment Tests: (*a*) From milk of low germ content; (*b*) from ordinary market milk rich in lactic acid *streptococci*; (*c*) from dirty market milk, with clumps of rod-shaped bacteria; (*d*) from gargety milk with many leucocytes and mastitis streptococci. Magnification, 700 diams.

specimens are reproduced in Fig. 33. The presence of many leucocytes and streptococci (chains of round or disc-shaped cells) associated together is a practically certain test for mastitis or garget. On the other hand, sometimes the milk of sound cows may show very considerable leucocyte separation in the sediment. But in such cases microscopic examination shows no streptococci.

If the number of mastitis streptococci in the sediment is small it may be increased by inoculating them into ordinary bouillon, or into bouillon to which has been added $\frac{1}{2}$–1 per cent. caffein (Baruchello, *Centralbl. f. Bakt.*, I. Abt., Orig., **39**, pp. 569–73).

For the more certain detection of mastitis milk, Russell and Hastings (*Experimental Dairy Bacteriology*, 1909, p. 122) used the following *test for "fibrin."* Flood the dried smear with aniline-

gentian violet (prepared in the same way as the aniline Victoria blue in § 35) and allow the stain to act for five minutes. Wash off and flood with Lugol's solution for two minutes. Treat with aniline oil till the colour is completely discharged. Wash with water, dry, and mount in Canada balsam. Fibrin should appear as threads stained dark purple.

References for §§ 49–51: The corresponding chapters in the text books quoted in paragraphs 4 and 6 on p. 2, especially Löhnis, *Handbuch der landw. Bakteriologie*, 1910, pp. 153–170, 175–179. The important question of leucocytes and streptococci is dealt with in detail by W. Ernst, *Monatshefte f. prakt. Tierheilkunde*, **20**, 1909, pp. 414 *et seq.*, **21**, 1909, pp. 63 *et seq.*

XII. REDUCTASE, CATALASE, BOILING AND ALCOHOL TESTS.

52. Reductase Test. Milk constituents, especially bacteria in milk, have the property of reducing or decolorising certain colouring materials, as methylene blue, indigo red, litmus, &c. In general, the more bacteria there are in milk, the quicker will methylene blue be reduced. Still, variable results may be obtained, as some kinds of bacteria have strong, while others have only weak reducing properties. The lactic acid bacteria belong to the latter class; but these organisms are generally not to be considered undesirable in milk. Good milk, therefore, takes a long time to reduce methylene blue; bad milk reduces quickly. In Sweden and Denmark the reductase test is already extensively used for the examination of market milk. Milk may be classified into the following four groups:

Time of reduction:	More than 7 hours,	7–2 hours,	2–½ hours,	less than ½ hour,
Quality:	good.	medium.	bad.	very bad.

But further experimental tests of this method are still required. These might be conducted in conjunction with the numbering of the bacteria, § 50. One cubic centimetre of diluted methylene blue (5 c.c. conc. alc. methylene blue and 195 c.c. dist. water) is added to 40 c.c. milk in large

test tubes (or fermentation tubes, *see* § 55) and the tubes kept in the water bath at 38°–40° C.

53. **Catalase Test.** Most substances of animal or vegetable origin, *e.g.* unpasteurised milk, and the bacteria which are found in the latter, have the power of splitting up hydrogen peroxide. Lactic-acid bacteria, however, and a few others of less importance, are exceptions in this respect,

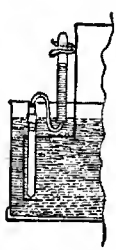


Fig. 34. Catalase apparatus, *after Henkel* ($\frac{1}{12}$ natural size).

Fig. 35. Catalase apparatus, *after Löhnis and Schröter* ($\frac{1}{4}$ natural size).

having little or no power of liberating oxygen from the peroxide. An enzyme called catalase is active both in milk and in bacteria. If 5 c.c. of a 1 per cent. solution of hydrogen peroxide (prepared by mixing one part of the so-called "Perhydrol" supplied by Merck of Darmstadt, with twenty-nine parts of distilled water) is added to 15 c.c. milk, under normal conditions, not more than 2–4 c.c. of oxygen are liberated in two hours at ordinary temperature (20°–22° C.).

A number of different forms of apparatus have already been recommended for the collection of the gas evolved, but none are

quite satisfactory. Two comparatively simple types which are convenient and fairly reliable are illustrated in Figs. 34 and 35. Henkel's apparatus, complete for fifteen to thirty samples, can be had from Wagner and Munz in München, Carlstr. 43. The apparatus used in the author's laboratory is so constructed that, once the water-level in the lower part has been brought to the zero mark, it can be used for a long time without any correction being necessary. The milk and hydrogen peroxide are filled into the tube M, which is placed in the apparatus, and both of the one-holed rubber stoppers put in their places, the stopper K being closed with a piece of glass rod. At the end of the experiment the stopper W may be closed in the same way to fix the height of the water in the graduated arm.

Colostrum and mastitis milk often give readings of 6–10 c.c. or more. The gas liberation is also greater, the larger the number of germs in the milk. Milk sterilised by heat has no catalytic reaction and hence any gas evolution in such cases indicates incomplete sterilisation. Examine different kinds of milk by this method.

54. **Boiling and Alcohol Tests.** Both tests give a general idea as to the degree of decomposition reached in a sample of milk, but no direct information as to the acidity. Neutral 70 per cent. alcohol is used for the alcohol test (2 c.c. mixed with 2 c.c. milk in a test tube). Forty-four per cent. alcohol gives approximately the same results as the boiling test. Good milk kept at a temperature under 12° C. for six days, or even longer, does not coagulate in either test.

The tests given in §§ 50–53 and also in §§ 55 and 56 should be applied to the same samples of milk, if possible.

XIII. MILK FERMENTATION AND CURD TESTS. DETECTION OF FOREIGN INFECTION.

55. **Milk Fermentation Test.** Forty to sixty cubic centimetres of milk are filled into large test tubes or special fermentation tubes which have been previously sterilised in the air oven, and these are kept for twelve to twenty-four

hours in a water bath at 40° C. A special apparatus may be used, such as that illustrated in Fig. 36.

The most important *types of fermentation* (illustrated on Plate II., Fig. 1) are the following:

1. Good milk remains unaltered.

2. Pure cultures of lactic acid bacteria produce a uniform, close curd without whey separation.

3. A gritty or cheesey curdling is produced by rennet-forming bacteria in conjunction, to some extent, with acid producers.

4. More or less gas development is observed in proportion to the number of dung bacteria (coli, aerogenes, and butyric bacteria, sometimes also yeasts).

5. When the udder is first affected with garget a *cream-yellow precipitate* is often visible in the milk (*see* § 51).

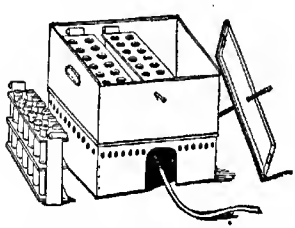


Fig. 36. Apparatus for milk fermentation test, about $\frac{1}{20}$ natural size.

Of course, all possible combinations of the above types may be found. After a little experience it is usually not difficult to form in this way a very correct opinion as to the quality of the milk. The taste and flavour of the milk must also be carefully tested. It should be noted that milk containing few germs often evolves some gas, but wrong conclusions as to the quality of the milk in such cases may be avoided by use of the reduction test (§ 52).

The combination of the reduction and the fermentation tests as recommended by O. Jensen has not proved satisfactory. The fermentation is often seriously changed by the addition of colouring matter.

The very objectionable flavour left occasionally on the palate by the fermented milk can be quickly destroyed by diluted perhydrol, which will also prevent infection from any pathogenic germs.

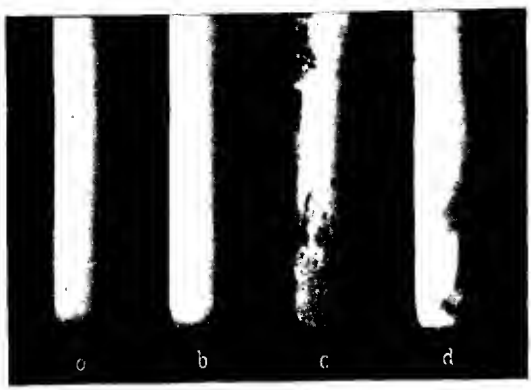


FIG. 1. Milk fermentation tests (§ 55): (*a*) gelatinous; (*b*) gritty; (*c*) cheesy; (*d*) blown.

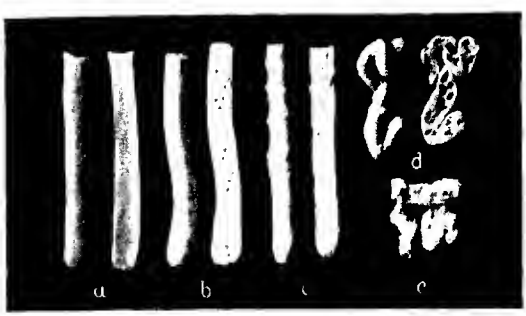


FIG. 2. Curd tests (§ 56): (*a*) smooth, without holes (8); (*b*) almost smooth, but apparently many holes (5); (*c*) rough, numerous holes (3); (*d*) irregular, spongy (1); (*e*) loose, torn (0).

56. **Curd Test.** A suitable quantity of rennet (*e.g.* 2 c.c. of a solution of rennet prepared by dissolving one of Hansen's rennet tablets in 500 c.c. water) is added to the milk in the tubes. The tubes are placed in the water bath at 40° C., and, after twelve hours, the resulting coagulum is examined. Important conclusions may be drawn from both the external appearance and the longitudinal section of the coagulum. These results supplement very well those from the fermentation tests. Illustrations of the most characteristic effects of the curd test are shown in Plate II., Fig. 2, opp. p. 68.

The figures in brackets in the descriptive note represent the "points" which the respective samples would be given in the fresh milk competitions of the German Agricultural Society. This society puts the greatest importance on these tests in conjunction with the boiling and alcohol tests.

References: Herz, *Jahrb. d. Deutsch. Landw-Gesellsch.*, **22**, 1907, p. 577; Löhnis, *Milchztg.*, **37**, 1908, p. 484.

57. **Detection of Foreign Infection.** When a milk fault[1] due to micro-organisms is met with the detection of the source of infection is of the greatest importance. Samples of milk should be taken at each of the various stages at which foreign infection may occur, *i.e.* from the earliest possibility of infection from gargety udder until the milk is in the vessels of transport. The samples are taken in sterilised tubes or flasks and kept at the temperature considered to be most *favourable* for the particular fault (certainly not always at 38°–40° C.). After twelve hours at most the source of infection should be located and preventive measures at once adopted. As a rule, little or no practical purpose is served by undertaking microscopic or special bacteriological investigations with milk sent from a distance, unless the samples supplied are taken in the manner described.

[1] It must, of course, first be ascertained whether the fault is infectious by inoculating it into sterilised milk and also into milk, unheated, but quite free from fault.

Investigate the effects of adding small quantities of earth, dung, straw, hay, water, &c., to milk and keeping it at different temperatures. The influence of the bacteria in the air of the cow-shed may also be tested either by exposing sterilised milk in sterilised vessels in the cow-shed or by sucking the air through sterilised milk in test tubes. If opportunity permits, samples of the milk in a dairy may be taken under aseptic conditions at various stages (udder, filter, milk vat, cooler, railway churns, etc.), and comparative experiments made. The detrimental influence of flies falling into the milk can also be demonstrated clearly by the fermentation tests.

XIV. BACT. COLI AND BACT. AEROGENES (AEROBACTER GROUP).

58. **Test for Bact. Coli.** The presence of both *Bact. coli* and *Bact. aerogenes* is, as a rule, due to particles of dung falling into the milk. Generally a very unpleasant flavour and odour in milk is caused by the presence of these organisms. In the fermentation test they may be readily detected by a vigorous development of gas (§§ 55 and 56). To arrive approximately at the number of these germs present in milk a series of dilutions may be tested in lactose bouillon and in æsculin bouillon (§ 49) at 38°–40° C. Evolution of gas in the former or the blackening of the latter indicates that their presence is probable; though not quite certain. If plates are poured with the same dilutions the percentage of these dung bacteria in terms of the total number may be estimated.

Besides *Bact. coli* and some other bacteria, yeasts and moulds are also able to blacken æsculin bouillon and to evolve gas from lactose. Again, every strain of Coli does not produce these effects, so that the results from these methods are not beyond criticism.

Water not above suspicion may be examined for Coli bacteria by putting a known quantity into milk-sugar bouillon (§ 49) and cultivating at 38° C. If gas formation

takes place further microscopic and cultural tests should be made.

59. **Isolating Bact. Coli and Bact. Aerogenes.** An inoculation is made from one of the fermentation tubes showing vigorous gas development (a little dung may have been previously added to the milk) into milk-sugar bouillon and this is kept at 38° C. As soon as strong evolution of gas is apparent, plate cultures are poured. The Aerogenes bacteria are non-motile and the colonies are easily recognised on flesh gelatine, being mostly round and porcelain-like in appearance. The colonies of motile Coli bacteria on the other hand are, especially in advanced growth, more leaf like and filmy. To make certain of securing the gas producers a whey agar tube is inoculated and poured into a Petri dish. When the agar has solidified, a second tube of agar is poured on the top to form a surface layer. The gas developed shows up the gas-producers quite distinctly. The number of varieties in the Aerobacter group is very large, while the kind and intensity of the gas production vary considerably.

Cultivate the species isolated on the six standard media (§ 4) and also in lactose and in æsculin bouillon, and make a shake preparation in whey gelatine (*see* Fig. 26, p. 43). One or more of the forms of apparatus described in § 32 can be used for fermentation tests. Make careful microscopic examinations.

XV. LACTIC ACID BACTERIA.

60. **Cultivation of Lactic Acid Bacteria (on Chalk Agar).** Sterilise three Petri dishes, and a test tube containing powdered chalk, in the air oven. Hold in readiness some soured milk and three whey-agar tubes. Put a small quantity of chalk into each Petri dish, about $\frac{1}{10}$ grm., and pour the plates in the usual way (§§ 21 and 34). The chalk added should make the agar only uniformly turbid, but not quite white. Mix it carefully with the agar and keep the plates, upside down, with glycerine paper, at 38° C. The

acid-producing colonies dissolve the chalk in their neighbourhood, and may be easily detected by the clear rings in the turbid agar (see Plate III., Fig. 1).

Chalky medium was first used by Beijerinck in 1891 (*Centralbl. f. Bakt.*, **9**, 1891, p. 781).

Examine the resulting colonies and make one or more inoculations on the standard media, on whey media, and lactose media (§§ 4 and 49). Pure cultures must be reinoculated at short intervals to prevent degeneration.

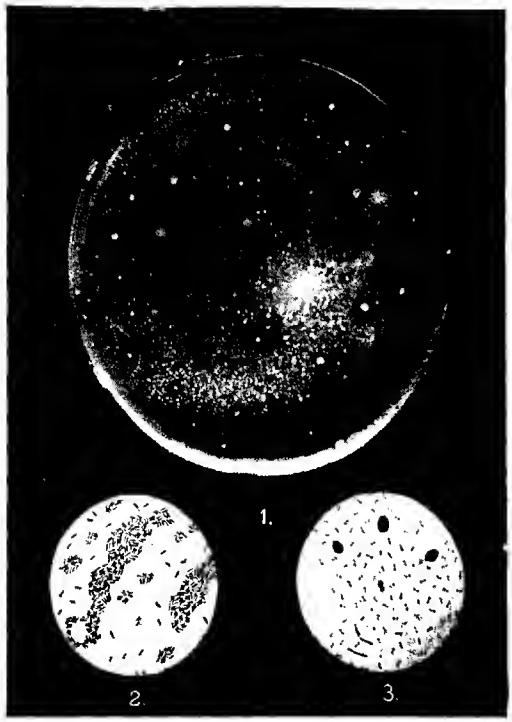
Microscopic preparations of soured milk stained in the usual way do not, as a rule, give good pictures because the precipitate is also coloured. Better results are obtained if the specimen is laid for five minutes in a small dish containing 5 c.c. chloroform and 1 c.c. conc. alcohol methylene blue, the chloroform poured off, and the specimen washed with water, dried, and mounted (*see* Utz, *Centralbl. f. Bakt.*, II. Abt., **11**, p. 611).

Chinese ink preparations are also recommended.

61. **Identification of Lactic Acid Bacteria.** Very many so-called different species of lactic acid bacteria are described in the different publications, but the different forms often resemble each other very closely and may in fact be regarded as merely varieties and not as separate species. The majority of these lactic bacteria which have, up to the present, been described may be conveniently divided into the following four groups :

1. **Intestinal Lactic Bacteria :** Thick, short rods ; grow better as aerobes ; optimum temperature between 35° and 40° C. The most abundant form is generally known by the name *B. aerogenes ;* the first variety to be thoroughly investigated was *B. acidi lactici*, Hueppe.

2. **Lactic Acid Streptococci.** Usually diplo- or streptococci in milk (*see* Fig. 33 *b*, p. 64) ; in flesh media often taken for little short rods ; generally grow better as anaerobes than aerobes ; the optimum temperature is mostly between 25° and 30° C. The most frequent form is *Streptococcus lactis*, formerly also called *Bact. lactis*, Lister, or *Bact. Güntheri*, Lehm. and Neum., or *Bact. lactis acidi*, Leichmann, etc.



1. Colonies of lactic acid bacteria on chalk agar (§ 60).
2. Microscopic specimens from Cheddar cheese, showing ripening centres (§ 79). × 600.
3. Microscopic specimen from Gervais cheese, showing an almost uniform distribution of bacteria (§ 79). × 600.

3. **Lactobacilli.** More or less long, thin to thread-like rods; grow better as anaerobes than as aerobes; optimum temperature is normally between 40° and 45° C. This form is most abundant in fermented milk (Yoghourt, etc.) and in cheese. It is called *Bact. caucasicum* or *B. casei*.

4. **Lactic Acid Micrococci.** Cells single or in irregular groups (not in chains); grow better as aerobes than as anaerobes; optimum temperature, generally at or under 20° C. The most abundant forms are *Micr. acidi lactis*, which liquefies gelatine, and *M. lactis acidi*, which does not liquefy gelatine.

With regard to other properties, as gas-production, liquefaction of gelatine, and the production of colour, the different varieties of each group may be arranged into several types (*see the* Identification Tables in Appendix I). Of course, the different types are connected by intermediate stages. For particulars as to the first group, *see* Chapter XIV. The lactobacilli, which can usually be isolated from market milk only by the use of special enriching methods, are treated more fully in Chapters XVIII and XXIII. Lactic acid micrococci are fairly common in milk which has been kept for some time (eight to fourteen days) at a low temperature (2°-5° C.), and they may also often be found in butter kept in cold storage (*see* Chapter XX). On the other hand, the lactic acid streptococci are regularly the most abundant in milk which has soured at ordinary temperatures.

In distinction to micrococci and those belonging to the Aerobacter group, the lactic acid streptococci and lactobacilli are together termed the "normal" lactic acid bacteria. Some other spore-bearing bacteria and vibrios are also capable of producing lactic acid, but they are, in practice, of no importance. Lactic-acid-producing yeasts can, however, take part in the ripening of cream (*see* § 75).

References: Löhnis, *Centralbl. f. Bakt.*, II. Abt., **18**, pp. 97-149 (review of bacteria already described—up to 1907—and their classification into groups and types); *ibid.*, **22**, pp. 553 and 554, and *Handbuch der landw. f. Bakteriologie*, 1910, pp. 192-202.

XVI. RENNET-PRODUCING BACTERIA. DISSOLVING OF CASEIN.

62. **Production of Acid, Rennet, and Proteolytic Enzymes.** Many lactic acid bacteria produce, besides acid, more or less rennet. Such organisms, called *Acid-Rennet-Producers* by Gorini, are specially numerous in the micrococci group, but other types, as streptococci, pigment-producing and colourless short rods, as well as spore-bearing bacilli, also often produce this double effect. The titration of curdled milk cultures shows whether the coagulation has been effected only by acid or by rennet, or whether both acid and rennet have been responsible.

Rennet producers almost always afterwards dissolve the coagulum more or less completely. Sometimes this process also takes place independently. As a rule gelatine liquefiers also dissolve casein and *vice versâ*, but there are exceptions to this rule.

63. **Cultivation on Milk Agar.** Add 3 per cent. agar to 100 c.c. tap water. After dissolving and filtering, fill 4–5 c.c. of the solution into each of a few test tubes and sterilise them. Keep the tubes of melted agar and the same number of milk tubes at 50° C. and bring some sterile Petri dishes to the same temperature. Empty one milk and one agar tube into each dish and mix thoroughly.[1]

The milk agar plates when solid are put into the incubator at 38° C. for some days to dry. Streaks are then made on this medium from pure cultures, or a cross may be made with two different organisms (for the study of symbiotic or antagonistic effects, *see* § 45). A white precipitate formed in the neighbourhood of the streak may be due either to acid or to rennet production; if due to acid, the addition of some weak soda solution will dissolve the precipitate, but if due to rennet the precipitate will remain. Proteolytic enzymes produce a clear zone.

Ordinary flesh agar, which contains sodium chloride,

[1] Milk and agar cannot be sterilised together. The milk would

mixed with 10 per cent. milk, also shows a partial clarification under the influence of acid producers. A little acid makes the casein soluble in common salt; while more acid precipitates it out again, a process which is of importance in the ripening of young cheese. A streak of *Streptococcus lactis* on milk-flesh-agar shows therefore an opaque border with a clear part beyond, while the proteolytic-acid-rennet-producers are surrounded by two clear rings separated by an opaque zone.

Rennet gives little or no visible effect on these milk-agar plates, because the milk is too much diluted.

References: Hastings, *Centralbl. f. Bakt.*, II. Abt., **12**, pp. 590 *et seq.* (Photograms), Van der Leek, *ibid.*, **17**, pp. 368 *et seq.*

64. **Cultivation on Casein.** In milk cultures of lactic acid bacteria, kept at 38° C. for four to six weeks and protected from evaporation by means of rubber caps (*see* § 14), all germs will have died (test by inoculation). These old cultures can be used to test acid destroying and proteolytic forms. Curd precipitated by rennet or acid may also be used. In the latter case the curd must be more or less freed from acid by washing with distilled water. After sterilising the curd, inoculate with the bacteria directly or after adding flesh-extract and (or) glucose to the medium.

References: Hueppe, *Methoden der Bakterien-Forschung*, 5 Aufl., 1891, p. 389; Grimm, *Centralbl. f. Bakt.*, II. Abt., **8**, p. 588.

XVII. PRODUCTION OF AROMA. SLIMY MILK.

65. **Aroma-Producing Bacteria.** Different species of the above produce a pleasant, fruity odour in milk. Pure cultures, however, often lose this property in a short time or, instead of the pleasant odour, produce foul-smelling, putrefactive substances. Of more practical importance (for cream and cheese ripening) are those species of lactic acid streptococci, lactobacilli, and even yeasts, which produce a desirable flavour in dairy products.

The specific aroma producers can be obtained from ordinary market milk by the method proposed by Van der Leek (*Centralbl. Bakt.*, II. Abt., **17**, p. 650). The milk is warmed to 30° C. in

Erlenmeyer flasks and ·01 per cent. rennet added. The mixture is then emptied into Petri dishes (to a depth of 1 cm.). It is kept at 30° C. till coagulation takes place and thereafter at 23° C. As soon as a distinct, fruity, odour is perceptible, generally after twenty-four hours, whey gelatine plates are poured. As a rule the aroma is caused by varieties of the *Aerobacter* group producing little or no acid and no gas. Micrococci, *B. fluorescens*, spore-bearing bacteria, etc., may also participate.

66. **Slimy Milk.** The majority of slime producers are closely related to the lactic acid bacteria. Slime production may also be caused by spore-bearing bacteria or by strains of *Oidium lactis*. Slime production may occur sometimes in any of the four groups of the lactic acid bacteria (slimy starters). Generally it is not very constant; the varieties which are most constant in this respect are certain micrococci, *e.g. M. pituitoparus*, Hohl. Milk kept for some time at a low temperature is most liable to become slimy. Often it is possible to enrich in these forms by adding 5 per cent. calcium chloride to milk, infecting with a little earth or dung, and keeping at 5°–15° C. for four to eight days.

67. **Staining Capsules.** Slime-forming bacteria are often surrounded by a more or less tough slimy capsule, which does not take on ordinary stains. In such cases the apparent sizes of stained and unstained bacteria differ widely. The capsules may be rendered visible in the following way (*after Johne*):

(1) Flood the fixed slide preparation with a 2 per cent. aqueous solution of gentian violet and warm till steaming.

(2) After washing with water, moisten with 2 per cent. acetic acid for six to ten seconds and again wash with water.

(3) Examine the specimen in water; Canada balsam, as a rule, makes the capsule disappear.

XVIII. FERMENTED MILK.[1]

68. **Number of Organisms.** In ordinary milk allowed to sour at about 20° C., by far the most abundant germs

[1] If the exercises, up to this point, have been carried out, the media prepared (§ 49) will be about exhausted. For the succeeding

are lactic acid streptococci. On the contrary *lactobacilli* predominate in the fermented milks fermented at 30°–45° C. (Koumiss, Yoghourt, Mazun, Dadhi, etc.), so common in eastern countries. *Yeasts* are also to be seen, but to a less extent (Fig. 37). It is only in Kefir, which is prepared at about 20° C., that the lactobacilli are more rare, and may even be entirely absent. In this case, along with strepto-

FIG. 37. Fermented milks. Magnification, 700 diams.
(*a*) Kefir, (*b*) Yoghourt, (*c*) Mazun, (*d*) Koumiss.

cocci and yeasts, some acid-producing, sporing bacteria are active. For preparing the best stained specimens see § 60.

References regarding Koumiss: Ruhinsky, *Centralbl. f. Bakt.*, II. Abt., **28**, pp. 161–219. *Yoghourt*: Kuntze, *ibid.*, **21**, pp. 737–768. *Mazun*: Düggeli, *ibid.*, **15**, pp. 594 *et seq*; Weigmann, Th. Gruber, and Huss, *ibid.*, **19**, pp. 70–87; *Dadhi*: Chatterjee, *Centralbl. f. Bakt.*, I. Abt., Orig., **53**, pp. 103–112. *Kefir*: Beijerinck, *Archives néerlandaises*, **22**, pp. 428 *et seq.*; Adametz, *Österr. Monatsschr. f. Tierheilkde.*, **15**, pp. 72 and 73; v. Freudenreich, *Centralbl. f. Bakt.*, II. Abt., **3**, pp. 47, 87, 135; Kuntze, *ibid.*, **24**, pp. 101–122.

69. **Cultivation of Lactobacilli.** Usually little or no growth is obtained on flesh media or on potato

experiments up to Chapter XXIII the following media are required: thirty tubes each of flesh gelatine, flesh agar, whey gelatine, and whey agar; fifteen tubes each of glucose agar, bouillon, milk sugar, bouillon, and potato slopes.

slopes. On the other hand, a comparatively vigorous growth is obtained in milk and whey media. This class of bacteria, the so-called *acidophile* bacteria, is able to thrive in presence of a relatively large amount of acid. For their cultivation bouillon with ½–1 per cent. acetic acid has been recommended. Better results are often obtained with milk or whey containing 1 per cent. peptone or 6–8 per cent. *yeast extract*. The latter is prepared, according to Rubinsky, in the following way: 100 grms. pressed yeast and 100 c.c. water are thoroughly mixed in a large beaker and heated in the steriliser for ½–1 hour. A large beaker is used to prevent frothing over. After being filtered two or three times through paper, the filtrate is sterilised in a small flask and kept for future use; ½ c.c. of this extract is added to milk or whey tubes. Yeast-whey is to be preferred for microscopic work. The lactobacilli from market milk, sauerkraut, silage, dung, earth, etc., can be relatively increased by two or three inoculations into this medium, and cultivating at 40° C.

The colonies of lactobacilli, which become apparent in the plate cultures at 38°–40° C. in three to four days, have sometimes the same form and size as colonies of lactic acid streptococci. Often they are more branched and appear like small knots of "tangled hair." At room temperature, 20° C., there is either no growth or only a very slow one. Chalk is dissolved as in the case of the streptococci. In most cases acidity develops less rapidly at first, but at the end of the experiment appreciably more acid is produced than in the case of the streptococci. Prepare some experiments on these lines.

70. **Cultivation of Milk Yeasts.** On the plates from fermented milk, yeast colonies are easily recognisable (very coarse granulation). It is more difficult to detect their presence in market milk, where they are almost always present in small numbers. They may be relatively increased according to the method of Troili-Petersson by adding 2 per cent. of a 3 per cent. solution of hydrogen peroxide (per-

hydrol ten times diluted) to the milk and incubating at 37° C. If the organisms being sought for are present, only a little, or no, acidity is developed, but instead a yeasty smell and fermentation is noticeable. Later on the casein may be dissolved. The isolation of the yeasts is often a very tedious business; the lactobacilli stick readily to the relatively large yeast cells, so that mixed colonies are persistently obtained. The one-cell method (§ 48) will be found helpful in this case (*see also* § 85).

The *iodoform reaction* commonly used for the detection of alcohol is as follows: The solution is first made neutral, and alcohol, aldehyde, acetone, etc., distilled over. The distillate (about 5 c.c.) is gently warmed and five or six drops of a 10 per cent. aqueous solution of caustic potash added, then a solution of iodine in potassium iodide drop by drop, till a brown colour is observed. The colour is removed with a drop of caustic potash. Alcohol, acetone, and aldehyde produce a smell of iodoform and the formation of hexagonal crystals of iodoform (examine under low power). Milk which has been sterilised in steam will also give this reaction. Control tests are necessary.

XIX. HEATED MILK. ANAEROBIC MILK BACTERIA.

71. **Peroxydase Reaction.** A large number of tests have already been recommended for detecting the destruction of oxydase and peroxydase in milk heated to 80° C. The modification of Storch's test given by Rothenfusser is very accurate. A mixture of paraphenylenediamine and guaiacol is prepared in the following way: 1 grm. of paraphenylenediamine chlorhydrate is dissolved in 15 c.c. of distilled water. To this is added 2 grms. of crystallised guaiacol in 135 c.c. of 96 per cent. alcohol (this solution should be kept in a dark-coloured bottle). The reagent is added to the milk, or, better, to the whey obtained by precipitating 100 c.c. milk with 6 c.c. lead acetate.

References: Rothenfusser, *Zeitschr. f. Unters. d. Nahrungs- und Genussmittel*, **16**, 1908, pp. 63–74; *Milchw. Centralbl.*, **6**, 1910,

p. 468; A. Hesse und Kooper, *Milchw. Centralb.*, **6**, 1910, pp. 412–420.

72. **Bacteria in Heated Milk.** It may be readily proved by the catalase test that there are often quite a considerable number of bacteria in the pasteurised and in the so-called "sterilised" milk on the market (*see* § 53). It should, however, be noted that deceptive results may be obtained in this way owing to the presence of organisms, like the butyric bacteria, which have no catalytic power.

The fermentation test (§ 55) can also be profitably used in a modified form for the examination of heated milk, especially for ascertaining the nature of the surviving bacteria. Put some milk into small flasks, stoppered by means of cap and spring arrangements as fitted on ordinary milk or beer bottles, after adding a little earth to make certain that spores are present. Heat some of the flasks for five to ten minutes to 70°–80° C., and the others for the same time to 100° C, and incubate at 38° C. Butyric fermentation will usually be found in the former, while in the latter the so-called "peptonising" or casein-dissolving bacteria will for the most part predominate.

73. **Cultivation of Milk Anaerobes.** Aerobic plate cultures from the fermentation test with heated milk will show various forms of the *subtilis* and *mesentericus* groups. In stained specimens from the same source, however, other interesting forms are to be seen which develop only under anaerobic conditions. Remember the instructions given in §§ 41–43, and make anaerobic cultures, using flesh and whey media. Refer to the Appendix I. (*Bacillus*) for the identification of obligate anaerobes. The non-motile butyric acid bacillus and *B. putrificus* are usually the more abundant. They have their origin in dung, but may be transferred from this source to the air of the cow-shed, where they can readily be detected by exposing Petri dishes containing sterile milk.

The number of anaerobes in clean milk is usually small. An approximately correct idea of the numbers in ordinary market

milk may be readily obtained by pasteurising in the above way (without addition of earth) and inoculating specific amounts of the milk, $\frac{1}{100}$, $\frac{1}{10}$, 1, 5 c.c., under aseptic conditions, into sterile milk tubes kept air-tight.

XX. GERM CONTENT OF BUTTER.

74. **Microscopic Specimens.** Butter of different ages, from sweet cream and from ripened cream, should be examined. In each case about 1 grm. is taken and mixed thoroughly in a small beaker or dish with a little (about 25 c.c.) warm water (60°–70° C.). This emulsion is centrifuged (*see* § 51), and from the sediment, which contains the germs, stained microscopic specimens are made.

In butter it is fairly common to find so-called "acid-fast" bacteria, which are remarkable in that they resemble in this respect the tubercle bacillus. They are detected by staining in almost the same way as for spores (§ 36) except that the treatment with warm carbol fuchsin is shortened to two minutes. The acid-fast bacteria (*i.e.* those which, like the tubercle bacillus, are not decolorised by acid alcohol) are seen as red rods among the blue cells.

75. **Number of Germs in Butter.** Generally from two to forty million germs per gramme are found in fresh butter. Sweet-cream butter usually shows an increase in the number of bacteria during the first days and weeks of storage, but thereafter the number decreases. In ripened cream butter there is a decrease from the first. Very old butter contains, as a rule, only a few hundred germs per gramme. Some butters are very rich in yeasts.

Make dilutions, according to the quality of the butter to be examined, and pour plates as in § 50. The water used for the dilutions should be previously warmed to 40° C. so that the butter (1 grm. in the first tubes or flasks) may be readily emulsified.

For the *sterile weighing* of butter, cheese, and similar substances, put a number of filter papers, 9 cm. and 7 cm. in diameter, into two Petri dishes respectively and sterilise in the air oven. By means of sterilised forceps, first place one

of the larger papers on the scale of the balance and then one of the smaller above it. After taring, with a sterilised knife or spoon place the substance to be weighed on the upper filter paper. Then transfer the material, along with the small filter paper, to the first dilution flask.

Count the number of colonies on the different media and examine later those organisms occurring most frequently.

76. **Examination of Starters.** When butter is made from acid cream the nature of the starter used has very considerable influence on the microflora, and in this way on the quality of the butter. The examination of starters should cover the following four points:

1. **Purity.**—Estimation of number and nature of organisms, on whey and flesh agar.

2. **Activity.**—Determine this at 20°–30° C., after having reinfected from the original material into sterilised milk on three consecutive days.

3. **Influence on flavour and aroma** of the cream and butter.

4. **Constancy of properties.**—Testing the production of acid, flavour, and aroma after progressive sub-culturing for a long time under favourable conditions.

Reference: Russell and Hastings, *Experimental Dairy Bacteriology*, 1909, p. 98 *et seq.*

XXI. FAT-SPLITTING GERMS.

77. **Enriching in Fat-Splitting Germs.** Dissolve 0·1 grm. KNO_3, 0·1 grm. NH_4Cl, and 0·05 grm. K_2HPO_4 in 100 c.c. tap water and divide into several small Erlenmeyer flasks in very shallow layers. After sterilising in the steamer add 0·3–0·5 per cent. of finely divided butyrin, or tripalmitin, or triolein, or swine fat, and inoculate with the material to be examined (butter, starter, water, &c.). Keep the flasks at 37° C. When growth begins inoculate into the

same culture solutions. For the microscopic observations, Chinese-ink preparations (§ 24) are best.

78. **Cultivation on Fat Agar.** For the detection of fat-splitting colonies, fat agar is used. This may be prepared in several ways. *Butyrin Agar* is made from flesh agar by adding 0·5 per cent. butyrin after sterilising. On this slightly turbid medium the colonies of the germs from the enriched cultures, or from rancid butter, generally cause local clarification similar to that by lactic acid bacteria on chalk agar.

The number of fat-splitting species is quite considerable. The decomposition of fat in butter, cheese, etc., is principally caused by *B. fluorescens* and its colourless, parallel form, *Bact. punctatum*, with the assistance of yeasts and moulds.

More particulars regarding rancidity in butter and other abnormal changes of butter are to be found in the author's *Handbuch der landw. Bakteriologie*, 1910, pp. 309–319.

XXII. GERM CONTENT OF CHEESE.

79. **Microscopic Examination.** The usual stained preparations from cheese do not give very satisfactory results. The shape as well as the position of the germs can be examined by cutting sections. But it is often rather difficult to get sufficiently thin sections, and better results are obtained in the following manner: A neatly shaped piece of cheese with quite even surfaces is placed between two clean object slides and these pressed gently together. The impress on the slide is fixed and stained in the usual way. The best results are obtained by using chloroform methylene blue (§ 60). Good specimens from hard cheese show clearly bacteria in localised groups which (by Rodella) have been called "ripening centres" (Plate III., Fig. 2, opp. p. 72). In soft cheese, especially when new, the bacteria are uniformly distributed (Plate III., Fig. 3).

80. **Numbering of Bacteria in Cheese.** The outer parts of young cheese usually contain many thousand millions, and the inside many millions, of germs per gramme. The maximum number is generally reached when the cheese is only a few days old, and sometimes even on the first day; as ripening proceeds the number becomes less and less. The numbers published in many papers are too small, on account of the difficulty of getting uniform division of the sample, and also, to some extent, because the methods used for cultivation are not quite suitable. One gramme of cheese, taken under aseptic conditions and weighed according to the method described in § 75, is ground in a sterile mortar with 10 c.c. of sterile tap water, and some sterile quartz sand or glass powder added, if necessary. The mixture is put into a dry, sterilised, 1000 c.c. flask, which is then filled to the mark with sterile water. After continuous shaking for several minutes, further dilutions with 9 c.c. water are made in test tubes; usually $\frac{1}{10.000}$ to $\frac{1}{1.000.000}$ is sufficient, only in the case of young cheese, as mentioned above, dilutions must be made to $\frac{1}{1.000.000.000}$. One cubic centimetre from these dilutions (*i.e.* $\frac{1}{100.000}$ to $\frac{1}{10.000.000}$, &c.) is run into each of three Petri dishes and mixed with agar or gelatine (*see* § 50). At the same time melted whey agar at 45° C. should be inoculated with these dilutions and filled into Burri tubes (*see* §§ 20 and 41). As the germs in cheese are generally best adapted for anaerobic conditions, the latter method, at 38°–40° C., as a rule, gives the higher numbers. Only the exterior of hard cheese, and flat soft cheese which, in contrast to the deep sorts, have an abundant air supply, contain more aerobic than anaerobic organisms. Reinoculate and identify the most frequently occurring germs.

A division of labour will be useful when counting the colonies.

More details on the micro-biology of the different bacterial species in cheese will be found in Part III, 3, of the author's *Handbuch der landwirtschaftlichen Bakteriologie.*

XXIII. LACTIC ACID BACTERIA IN CHEESE. ANAEROBIC BACTERIA IN CHEESE.[1]

81. **Lactic Acid Bacteria in Cheese.** The majority of germs in cheese are lactic acid bacteria. Representatives of each of the four groups (§ 61) may be found. The intestinal lactic acid bacteria are rare in good cheese, as in clean milk, but in faulty, and especially in gasey cheese, they are generally very numerous. *B. aerogenes* and related forms play an important *rôle* in this gas production. Lactic acid micrococci, since they require a fair amount of air, predominate in the outer parts of the cheese. Casein-dissolving varieties from this group are very common in the rind of young cheese. However, by far the more frequently found are streptococci and lactobacilli, and particularly the latter in hard cheese made with lactobacilline starter. Direct inoculation of whey agar contained in Burri tubes with Emmenthal cheese usually gives large numbers of the characteristic "hair tangles"; with other cheese it is better to first increase the number of the lactobacilli by cultivating in yeast extract whey (*see* § 69).

O. Jensen (*Centralbl. f. Bakt.*, II. Abt., 4, p. 196) recommends peptonised milk for the cultivation of lactic acid bacteria in cheese. This medium is prepared thus: To one litre sterilised milk add 10 c.c. pure conc. hydrochloric acid (33 per cent. HCl) and 2 grm. pure pepsin (pepsin. German, pur. gran.). Keep the mixture for thirty-six to forty-eight hours at 35°–37° C. and shake frequently. Neutralise, and heat in the autoclave to 115°–120° C. for ten minutes. After filtering, so regulate the acidity (using phenolphthalein as indicator) that 5 c.c. neutralises 1–2 c.c. N/10 soda. After clearing with albumen, filter again and add gelatine or agar if desired; of course, the reaction in gelatine must be corrected.

Casein-dissolving types are especially abundant in the lactic acid micrococci group, but some of the streptococci

[1] Before beginning, prepare the media required for the exercises in Chapters XXIII and XXIV (*see* § 49 and Chapter XVIII, Note 1).

and lactobacilli also possess this property. Examine the isolated types on milk agar, and perhaps also on casein (*see* §§ 63 and 64).

82. **Anaerobic Bacteria in Cheese.** With few exceptions, quite as few obligate anaerobes are found in cheese as in milk. One exception is the "Schabzieger" (Kräuter cheese) in the ripening of which the strictly anaerobic butyric acid bacteria take a distinct part. On the other hand, the albuminoid decomposers from the same family as *B. putrificus* are comparatively rare and of little importance.

The anaerobic bacteria can be relatively increased by the method given in § 72, with appropriate modification (sterilised milk inoculated with cheese instead of with earth). The albuminoid decomposers are secured by putting the cheese emulsion, under anaerobic conditions (§ 42), in a test tube containing a piece of hard-boiled white of egg and four to five times this volume of water. False conclusions may, however, be drawn from this experiment as the enzymes present may also dissolve the albumen.

83. **Formation of Volatile Fatty Acids and Gases.** The flavour of dairy products is caused partly by the not inconsiderable amount of free fatty acids produced by fat-splitting organisms. The isolation of these is described in § 78. Besides these the decomposition of albuminoids and the fermentation of lactates, succinates, glycerine, etc., must be considered. Gas production in hard cheese is chiefly due to these fermentations. The organisms causing these changes may be relatively increased by using the following solution (O. Jensen): 100 tap water, 2 pepton (Witte), 0·5 NaCl, 0·2 KH_2PO_4, 0·05 $MgSO_4$, and 2 Ca-lactate or 1·0 Ca-succinate or 5 glycerine. After repeated progressive sub-culturing, isolation may be accomplished on whey agar.

Reference for §§ 81–83: Lõhnis, *Handbuch der landw. Bakteriologie*, Teil III., 3 Abschnitt A, Kap. 3 and Abschnitt B, Kap. 4.

XXIV. LIQUEFYING BACTERIA. YEASTS AND MOULDS.

84. **Liquefying Bacteria.** Besides the lactic acid bacteria, other kinds are found, especially in the margins of cheese, which dissolve casein and generally also liquefy gelatine. If the dissolving of the casein proceeds in a weakly acid medium, the so-called *acid-rennet-bacteria* of Gorini are responsible. They are in some respects similar to the casein-dissolving lactic acid bacteria (*see* § 62). The bacteria colouring the rind of soft cheese, *red or brown*, also dissolve casein, but not all of them liquefy gelatine as well.

The vigorous gelatine-liquefying, aerobic, spore-bearing bacteria from the group *B. subtilis* and *mesentericus*, formerly called *Tyrothrix*, which figured in text books as important ripening agents, are really of subordinate importance. Special enriching methods must be adopted to discover them in cheese. Sugar-free bouillon (made according to § 32) is inoculated with the cheese emulsion and pasteurised for five to ten minutes at 80° C., and flesh gelatine and flesh agar plates are poured.

85. **Yeasts.** While, in hard cheese, yeasts are usually found in relatively small numbers, in soft cheese they play a more or less important part. Gervais, Imperial, and similar cheeses, being specially rich in yeasts, make excellent material for the study of these organisms. In Cheddar cheese special yeasts are sometimes the cause of faulty ripening, disagreeable flavour, as well as of "blowing."

Plate cultures with practically only yeasts present may be made in the following way (Russell and Hastings): Dissolve in a test tube 1 grm. tartaric acid in 10 c.c. water, sterilise, and transfer 1 c.c. of the solution (by means of a sterile pipette) to a Petri dish. Pour the inoculated flesh agar, and mix thoroughly with the acid; this will prevent almost all other organisms from growing. If a preliminary enriching seems necessary, proceed as directed in § 70.

The identification of cheese yeasts is not easy; thorough comparative experiments are needed. Besides the references quoted in the author's *Handbuch der landw. Bakteriologie*, see a research of later origin by W. Dombrowski (*Centralbl. f. Bakt.*, II. Abt., **28**, pp. 345-402.

86. **Moulds.** Moulds are of special importance in cheeses of the Brie, Camembert, and Roquefort type. Usually they grow well on the ordinary plate cultures.

Dishes, tubes, and flasks containing moulds must be opened with the greatest care in order to prevent the air of the laboratory being infected.

The following are a few of the large number of special media for moulds.

Bread mash, prepared from dry crumbled bread, filled into Erlenmeyer flasks, soaked with water, and sterilised three times in the steriliser.

Rice mash, prepared from 10 grm. rice meal, 15 c.c. milk, and 5 c.c. bouillon, and sterilised in the same way as bread mash.

Beer wort agar, prepared by adding 1½ per cent. agar to beer-wort, without neutralising.

Plum extract gelatine, prepared by boiling 100 grms. of dried plums twice with 100 c.c. water. To the 200 c.c. plum extract obtained, 10 per cent. gelatine is added, without neutralising.

C. BACTERIOLOGY OF MANURES.

XXV. GERM CONTENT OF FARMYARD MANURE.

87. **Culture Media.** Two hundred and fifty test-tubes are made ready for use, and the following media prepared as in §§ 6–13:

40 tubes of flesh gelatine.	20 tubes of bouillon.
40 tubes of flesh agar.	20 tubes of milk.
20 tubes of glucose agar.	20 tubes with potato slopes.

Dung extract is also required. Take about 150 grms. of dung, extract for twenty-four hours with 300 c.c. water, and filter through folded filter paper. Add 0·05 per cent. K_2HPO_4. To 100 c.c. add $1\frac{1}{2}$ per cent. agar, and to 100 c.c. 10 per cent. gelatine, then neutralise, clarify the latter with white of egg, and fill into tubes.

This stock of media should suffice for the exercises in Part C, provided more detailed investigations are not made.

Particulars as to special media will be given later.

88. **Microscopic Examination.** Make hanging-drop and other slide preparations from fresh excrements and from rotted dung (after thorough mixing with a little water if necessary). Unstained smear preparations from solid excrements, mounted, after drying, in Canada balsam, give most interesting pictures (Fig. 38). Masses of different bacteria are seen between the undigested food particles. Most varied observations are provided by the hanging-drop preparations from decomposing liquid manure. Spirilla, which are difficult to cultivate, are generally seen in dainty, screw-like, motion.

Stained slide preparations may be used for the microscopic numbering of bacteria, after the following method recommended by A. Klein: One gramme of solid excrement is vigorously shaken with 4 c.c. sterilised water in a test tube. By means of a pipette ½ c.c. of the turbid liquid is transferred to a watch glass, and ½ c.c. aqueous aniline Victoria blue added (§ 35). After thorough mixing, a known amount is removed with the platinum loop, and spread uniformly on a known area of the slide or cover glass. To ascertain the weight of liquid removed by the platinum loop, several loopfuls must previously be taken, weighed, and averaged. The liquid on the slide is allowed to dry, fixed, and mounted in Canada balsam (naturally without washing). Fifty fields are then counted, the area of the field measured, and the total number of germs reckoned.

FIG. 38. Unstained specimen of cattle fæces. Magnification, 700 diams.

Of course, the experimental error of this method is very wide. But it gives a more correct idea of the number of germs in dung than can be obtained by plating. Also since dead bacteria take on less colour a rough idea can be got as to the proportion of living to dead germs present in the excrements.

References: A. Klein, *Centralbl. f. Bakt.*, I. Abt., **27**, p. 834; *Archiv. f. Hygiene*, **45**, 1902, pp. 122 *et seq.*

89. **Aerobic and Anaerobic Plate Cultures.** Sterilise in the autoclave or in the steamer one litre of water in a 2-litre flask, six test tubes (each with 9 c.c. water), eight 1 c.c. pipettes, and six Burri tubes; and in the air oven twelve empty Petri dishes and two with filter paper (as in § 75). Melt three tubes each of flesh and dung extract gelatine, and six tubes each of flesh and dung extract agar, and keep them in water at the proper temperature. Weigh 1 grm. of the substance (as in § 75) and shake it thoroughly for several minutes with the litre of sterile water. Make dilutions in the usual way in five tubes, shaking each tube

thoroughly[1] before measuring the 1 c.c. From each of the third, fourth, and fifth tubes, corresponding to dilutions of $\frac{1}{1.000.000}$ to $\frac{1}{100.000.000}$, 1 c.c. is transferred into the labelled dishes. Inoculations are also made into three flesh agar and three dung extract agar tubes for anaerobic cultivation. The platings are completed, incubated, and numbered in the usual way (§§ 17, 20, and 34). The numbers found are, as a rule, far below the numbers which really exist in dung. In 1 grm. of solid excrement 2000 to 20,000 millions are often present.

Inoculate from the most frequently occurring colonies into fresh media, plate again, and investigate the pure cultures obtained, in the usual way (Ch. V.–VIII.).

90. **Gravimetric Estimation of the Organisms in Fæces.** This can be done by the method of J. Strasburger by separating both living and dead germs from the dung particles with the centrifuge. The method which has been often tested and modified specially for the examination of human fæces is as follows: Mix 2 c.c. solid excrement in a mortar with 70 c.c. of ¼ per cent. hydrochloric acid, divide into two portions—35 c.c. in each—and centrifuge rapidly for five minutes (about 2000 revolutions per minute). Pipette off the clear liquid, add two parts 96 per cent. alcohol, and keep at 38° C. for twelve to fifteen hours. Centrifuge this liquid and wash the precipitate obtained with alcohol, then treat with ether, and allow to stand for one day, but shake frequently. Syphon off the clear liquid, add alcohol, centrifuge, and treat the residue with pepsin-hydrochloric acid for one day. Dilute the solution with two parts of alcohol, centrifuge for the last time, wash the sediment with alcohol into a porcelain dish, dry and weigh.

References: Strasburger, *Zeitschr. f. klin. Medizin*, **46**, 1902, p. 418; Ermann, *Über eine Methode zur Feststellung der in den Fäzes enth. Bakt.* Diss. med. Bonn, 1902; Ehrenpfordt, *Zeitschr. f. experim. Pathologie*, **7**, 1909, p. 455.

[1] As shown by direct microscopic examination the germs are often present in clumps which can be divided only by vigorous shaking. If the tubes are not sufficiently shaken mixed colonies will result.

XXVI. ANAEROBIC DECOMPOSITION OF PECTIN AND CELLULOSE.[1]

91. **Anaerobic Pectin Decomposition.** The active organisms (belonging to the group of *B. amylobacter*) may be relatively increased in the following solution :

100 tap water.	0·05 K_2HPO_4.
0·05 $(NH_4)_2SO_4$.	2·00 $CaCO_3$.

About 20 c.c. of this solution is filled into each of three or four test tubes to which 0·1 grm. pectin [2] has previously been added.

After being sterilised in the steamer, one of the tubes is infected with a little rotted dung and incubated at 38° C. As soon as vigorous gas formation is observed, usually after four to eight days, a second tube is inoculated from the first, and so on ; by making further inoculations into fresh tubes the pectin decomposers are selected, and each time purer cultures are obtained.

The pectin decomposers should be carefully examined under the microscope, preferably in Chinese-ink preparations. They can be isolated, in Burri tubes, on agar prepared from diluted beer-wort weakly acid.

[1] The aerobic decomposition of pectin and cellulose is dealt with in Ch. XXXIII.

[2] Pectin is best obtained in the following way (Behrens and Störmer): 500 grms. roots or carrots are grated and extracted several times with warm water containing chloroform. The residue is treated for half an hour (without warming) with dilute caustic soda, washed with water, then extracted for half an hour with dilute hydrochloric acid and again washed with water. Finally, the pectin is dissolved by adding dilute ammonia and precipitated from the ammoniacal solution with ½ per cent. calcium chloride solution. The precipitate is well washed, finally on the filter paper, with much distilled water. Pectin can also be prepared from flax, hemp, straw, etc., but only in small quantities.

92. **Anaerobic Cellulose Decomposition.** The best known anaerobic cellulose-decomposers are the hydrogen and methane bacilli studied in detail by Omelianski. They may be cultivated in the following solution :

100 dist. water.	0·05 $MgSO_4$.
0·1 $(NH_4)_2SO_4$.	Trace NaCl.
0·1 K_2HPO_4.	2 $CaCO_3$.

Fill each of several tubes with about 20 c.c. of the solution and add about 0·1 grm. filter paper, cotton-wool, or straw. Infect the tubes richly with rotted dung and incubate at 38° C.

The *methane bacillus* is relatively increased by sub-culturing in the same solution as soon as the fermentation begins (without pasteurising). The *hydrogen bacillus*, on the other hand, is obtained by first pasteurising before each reinoculation so that the methane bacilli, at this time in the vegetative form, are killed and the hydrogen bacilli in spore form are carried over.

These anaerobic cellulose decomposers have only in exceptional cases been grown on solid media.

References: Omelianski, *Centralbl. f. Bakt.*, II. Abt., **8**, specially pp. 226 *et seq.*, 263, 290 *et seq.*, 355 *et seq.*, and **11**, p. 369. With regard to other cellulose-decomposers see the author's *Handbuch d. landw. Bakteriologie.*

XXVII. GAS FORMATION. THERMOPHILE BACTERIA.

93. **Production of Methane.**[1] The methane evolved along with CO_2 and H from a dung heap does not arise only from the fermented cellulose but also from the decomposition of salts of organic acids, from gum, starch and sugar, and even from albuminoids. The following solutions may be used :

100 tap water,
·05 NH_4Cl,
0·05 K_2HPO_4,
} + 1 per cent. { Acetate, butyrate, lactate, etc.,
Gum, starch, sugar, peptone, or wool.

[1] These experiments on the production of CH_4 and H may be combined with those in Chapter XXXIV on the assimilation of these gases.

These solutions are filled into test tubes, richly infected with rotted dung, and incubated at 38° C.

The causal organisms have not yet been completely investigated. Chinese-ink preparations yield very interesting pictures.

References: Mazé, *Compt. rend.* (Paris), **137**, p. 887; Omelianski, *Centralbl. f. Bakt.*, II. Abt., **15**, p. 679; Söhngen, Proefschrift Delft, 1906, ref. *Botan. Centralbl.* **105**, p. 371.

94. **Production of Hydrogen.**[1] As in the case of methane, the hydrogen developed from dung may have other sources besides cellulose decomposition. Hydrogen is set free in greater or less amount by the numerous types of *Aerobacter* and *Amylobacter*, which are commonly found in large numbers in rotted dung. The gas may also arise from salts of organic acids, starch, sugar, or even from albuminoids, along with CO_2. A H-producing variety of *B. coli* named *Bact. formicicum* may be relatively increased in the following solution: Tap water + 0·2 per cent. peptone + 2 per cent. calcium formate, filled into test tubes, infected, and incubated for some days at 38° C.

Reference: Omelianski, *Centralbl. f. Bakt.*, II. Abt., **11**, pp. 177, 256, 317, and **14**, p. 674.

95. **Production of Carbon Dioxide.** The great majority of the germs in dung give out carbon dioxide. The intensity of the development may be easily tested. Air, free from carbon dioxide, is sucked through dung in a flask, fitted with a two-holed rubber stopper and suitable absorption tubes; for demonstration purposes use absorption tubes filled with baryta water; for quantitative estimation use Liebig's caustic potash apparatus.

96. **Thermophile Bacteria.** On account of the very vigorous decomposition in dung, especially during the first weeks, the temperature frequently exceeds 45°–50° C. At this temperature only the thermophile bacteria remain active. To isolate these germs infect bouillon with dung, and incubate at 50°–60° C. Prepare agar plates and keep them, after solidification, at the same temperature.

[1] *See also* § 132, p. 116.

XXVIII. DECOMPOSITION OF UREA, HIPPURIC ACID, AND URIC ACID.

97. **Urea Bacteria.** Prepare 500 c.c bouillon and take 200 c.c. from it for making flesh gelatine (adding 12 per cent. instead of 10 per cent.) and 200 c.c. for flesh agar. To 50 c.c. of the remaining 100 c.c. bouillon add 2 per cent. urea, and to the last 50 c.c. 10 per cent. urea. Fill the different media into test tubes and sterilise them in the usual way. Then add 1 c.c. of a 15 per .cent. aqueous solution of urea to each of eighteen gelatine and twelve agar tubes, and heat these once more in the steam steriliser.

The addition of urea reduces the solidifying power of the gelatine and it is therefore not added until just before the last sterilisation. A small amount of urea is converted into ammonia by heating in the steamer, but this has little influence on the experiment. Of course, heating in the autoclave must be avoided. The gelatine and agar tubes to which urea has not been added complete the stock.

One of the bouillon tubes with 2 per cent. urea is infected with solid or liquid manure and incubated at about 10° C. In this, various short rods and micrococci develop. One of the other bouillion tubes with 10 per cent. urea is infected with the same material and kept at 38° C. In two to four days a strong odour of ammonia is perceptible, and long spore-bearing bacteria are to be found in the slightly turbid solution.

As soon as there is a distinct odour of ammonia in both tubes, re-inoculations are made into fresh culture solutions of the same kind and kept under similar conditions. From the 2 per cent. urea bouillon, platings are made with urea gelatine; from the 10 per cent. urea bouillon, with urea agar. The isolated pure cultures should be tested on the six standard media as well as on urea agar, urea gelatine, and urea bouillon. The forms isolated from the 10 per cent. urea bouillon hardly ever grow on the standard media, while those from the 2 per cent. urea bouillon often do grow.

The most frequently occurring sporing bacterium on the 10 per cent. medium is *Bacillus Pasteurii*; on the other, *B. fluorescens*, micrococci, and different varieties of *Bact. coli, vulgare, erythrogenes*, etc., are found.

Crystal formation is fairly common in urea media, but it does not occur so regularly as to be a certain indication of the presence of bacteria which split up urea.

References: Beijerinck, *Centralbl. f. Bakt.*, II. Abt., **7**, p. 33. Particulars regarding other culture solutions, especially for enriching in urea decomposing bacteria from soil, are given in § 119.

98. **Hippuric Acid Bacteria.** Ten cubic centimetres of the following solution: 100 c.c. tap water + 0·3 grm. sodium hippurate + 0·05 grm. K_2HPO_4—is filled into small Erlenmeyer flasks and infected with solid or liquid manure. The fermentation is rather slow. Micrococci, various short rods (*Bact. prodigiosum, vulgare*, etc.), and also moulds take part.

99. **Uric Acid Bacteria.** Ten cubic centimetres of the following solution: 100 c.c. tap water + 0·3 grm. uric acid + 0·05 grm. K_2HPO_4 is filled into small Erlenmeyer flasks and infected with some solid or liquid manure. The fermentation is due to *B. fluorescens* and other bacteria, and also to moulds. Often, but not always, the urea formed as an intermediate product in the process is decomposed by the same organism. For isolating, flesh gelatine may be used.

XXIX. NITRIFICATION. DENITRIFICATION. REDUCTION OF NITRATES.

100. **Nitrification.** In dung considerable numbers of nitrifying bacteria are usually found only in the surface layer of heaps, and at the bottom of shallow stalls. In the deeper layers of the manure heap, and in deep stalls, the growth of these organisms is prevented, principally by want of air and by the presence of free ammonia. The natural home of nitrifying bacteria is in arable land.

Since the nitrification experiment at ordinary tempera

ture extends over three to four weeks, the following three solutions may advantageously be infected with both dung and earth at the same time. The isolating of the bacteria from these cultures may be undertaken later (§ 121). By cultivating at 25°–30° C. the work can be considerably hastened. The first of the following three solutions is adapted for relatively increasing the nitrite bacteria, the second the nitrate producers, and the third the simultaneous growth of both organisms, as in nature.

I.		II.
100 dist. water,		100 dist. water,
0·10 $(NH_4)_2SO_4$,		0·10 $NaNO_2$,
0·10 K_2HPO_4,	Basic magnesium carbonate in excess after sterilising.	0·05 K_2HPO_4,
0·05 $MgSO_4$,		0·03 $MgSO_4$,
0·20 NaCl,		0·05 NaCl,
0·04 $FeSO_4$,		0·03 Na_2CO_3.

III. The same solution as I., but instead of magnesium carbonate, calcium carbonate is added after sterilising.

Ten cubic centimetres of each are filled into 50 c.c. Erlenmeyer flasks and infected in the one series with about $\frac{1}{10}$ grm. dung and in the other with $\frac{1}{10}$ grm. rich soil. After eight to fourteen days, the solution, which remains clear, should be tested every second or third day by transferring a loopful with a sterile platinum loop to a white plate or tile, and testing with an "outside" indicator. The disappearance of ammonia is tested with Nessler's solution, the production or disappearance of nitrite with naphthylamine-sulphanilic acid. Diphenylamine-sulphuric acid shows both nitrite and nitrate.

The solution for testing for nitrite is prepared in the following way: 0·5 grm. sulphanilic acid is dissolved in 150 c.c. 33 per cent. acetic acid and 0·1 grm. α-naphtylamine in 20 c.c. warm water. To this latter solution is also added 150 c.c. 33 per cent. acetic acid. The two solutions are mixed and kept in an air-tight bottle. This reagent gives a red colour in the presence of 0·0002 per cent. of mg. nitrite.

101. **Denitrification.** The most frequently used culture solutions [1] are *Nitrate bouillon* and the so-called *Giltay's solution.*

The former is prepared by adding to a few (about six) bouillon tubes 1 c.c. of a 1 per cent. solution of sodium nitrate (corresponding approximately to 0·1 per cent.) and sterilising again. Ten flesh agar tubes should receive the same addition. The following represents Giltay's solution :

0·2 grm. KNO_3, 0·1 grm. asparagin,	Dissolved in 25 c.c. dist. water.	The two solutions are mixed, and made up to 100 c.c.
0·5 grm. citric acid, 0.2 grm. KH_2PO_4, 0·2 grm. $MgSO_4$, 0·02 grm. $CaCl_2$, Trace Fe_2Cl_6,	Dissolved in 50 c.c. dist. water and neutralised with caustic potash.	

35 c.c. are portioned out into four test tubes and sterilised; 1½ per cent. agar is added to the remainder, which is then filtered, filled into tubes and sterilised.

Put ¼–½ grm. of rotted dung into each of the above culture solutions. After two to three days at 38° C. frothing takes place. Re-inoculate each into the corresponding solution, and repeat the sub-culturing once or twice. Make plate cultures with nitrate agar in the one case and Giltay's agar in the other. After solidification, pour another agar tube on the top. In a few days at 38° C. the denitrifying colonies are recognisable by the development of gas. Cultivate these further on the standard media.

Straw decoction + 0·1 per cent. nitrate also favours vigorous denitrification when infected with dung.

The crystals deposited in Giltay's solution are magnesium phosphate (*see* Hutchinson, *Centralbl. f. Bakt.*, II. Abt., **16**, p. 326).

102. **Nitrate Reduction.** In nitrate bouillon the denitrifying bacteria sometimes may be completely crowded out by bacteria

[1] Further recipes are given in § 122.

which either reduce the nitrate to nitrite and to ammonia, or assimilate it, forming organic compounds. For the nitrite and ammonia tests *see* § 100, and for the nitrate assimilation § 105.

On the plates the colonies of the nitrate reducing bacteria may be detected by Beijerinck's method (*Centralbl. f. Bakt.*, II. Abt., **1**, p. 58). Besides nitrate 0·5 per cent. starch is also added to flesh agar and one half of the developed plate moistened with a solution of hydrochloric acid and potassium iodide. The reducing colonies become surrounded with a blue ring. Since the hydrochloric acid kills the bacteria the corresponding colonies for inoculation must be sought for in the other half of the plate.

XXX. ASSIMILATION OF AMIDE, AMMONIA, AND NITRATE.

103. **Amide Assimilation.** Prepare the following standard solution :

1000 c.c. dist. water,	0·02 grm. NaCl,
0·5 grm. K_2HPO_4,	Trace Fe_2Cl_6.
0·02 grm. $MgSO_4$,	

One hundred cubic centimetres of this solution is measured out four times separately, and to each 100 c.c is added 1 per cent. glycerine and 0·1 per cent. of one of the following nitrogen sources : urea, sodium hippurate, uric acid, or asparagin. Ten cubic centimetres of each mixture is put into each of the 50 c.c. Erlenmeyer flasks ; one series is infected with dung and another with earth. One and a half per cent. agar is added to the remainder of the different solutions and the media are used for the first isolations. For further examinations the standard media may be used. Various sporing and non-sporing bacteria (*Bac. pumilus, Bact. fluorescens, radiobacter, aerogenes, erythrogenes*, etc.) as well as yeasts and moulds may take part in amide assimilation.

Instead of glycerine many other sources of carbon may be used, *e.g.* cane sugar, glucose, mannite, calcium lactate, etc., each of which results in corresponding variation of the microflora.

The albuminoid protection in the digestion process afforded by some amides in food stuffs may be demonstrated in those cases where the amide-assimilating bacteria dissolve casein by making a stab culture with the particular bacteria in milk agar, § 63, and in milk agar to which 0·1 per cent. asparagin has been added. In the former medium clarification circles are seen, but not in the latter.

104. **Ammonia Assimilation.** One per cent. glycerine or 1 per cent. glucose is added to 100 c.c. of the standard solution (§ 103), and also 0·1 per cent. of one of the following nitrogenous substances: ammonium sulphate, ammonium acetate, ammonium butyrate, or ammonium lactate. The instructions given in § 103 should be modified accordingly.

Different sources of carbon in this case also produce different results.

105. **Nitrate Assimilation.** Add 0·1 per cent. nitrate of soda and either 1 per cent. glucose or 1 per cent. glycerine to 100 c.c. of the standard solution (§ 103). Proceed as in § 103. With glucose almost nothing but moulds develop; with glycerine, various bacteria, and usually some denitrifiers.

References for §§ 103–105: Bierema, *Centralbl. f. Bakt.*, II. Abt., 23, p. 672. (Detailed examination of a large number of sources of nitrogen and carbon and of assimilating organisms.)

D. SOIL BACTERIOLOGY.

XXXI. GERM CONTENT OF SOIL.

106. **Culture Media.** Two hundred and fifty test tubes are made ready for use, and the following media prepared from 1500 c.c. bouillon according to the instructions given in §§ 6–13.

70 tubes flesh gelatine,	30 tubes bouillon,
30 tubes flesh agar,	30 tubes milk,
30 tubes glucose agar,	30 tubes with potato slopes.

The remaining bouillon is sterilised in a suitable flask and reserved for later use.

Soil Extract is prepared in the following way: One kilo. of good rich garden soil is either heated with one litre of tap water for half an hour in the autoclave under pressure of 1 atm. or boiled with two litres water for two hours over the free flame. The turbid liquid is then poured off, some talc mixed with it, and the mixture filtered through a double filter paper. The first filtrate, which is generally turbid, is poured back on to the filter. One kilo. of earth should yield about 800 c.c. extract, but if it does not, owing to loss of volume during boiling, the filtrate is made up to 800 c.c. Two volumes of 100 c.c. each of the extract are used, as with bouillon, to prepare *Soil-Extract Gelatine* and *Soil-Extract Agar*, ten to twelve tubes of each. The remainder is sterilised in a flask and reserved for future use.

This stock of media, with the exception of the flesh gelatine and the flesh agar, should be sufficient for the exercises in Part D, provided more detailed investigations are not undertaken. Particulars regarding the preparation of some special media are given later.

107. **Germ Content of Soil.** By shaking up some soil in water and making a Chinese-ink preparation (§ 24), it is at once seen that the number of bacteria in soil is relatively small. Plate cultures are made in the usual way: 1000 c.c. tap water in a 2-litre flask, five test tubes with 9 c.c. water each, and six 1 c.c. pipettes are sterilised in the autoclave or in the steriliser, and twelve empty Petri dishes, and if necessary, two with filter paper (*see* § 75), in the air oven. Flesh gelatine, soil extract gelatine, flesh agar, and soil extract agar tubes, three of each, are melted, cooled, and held in readiness in water at the proper temperature. One gramme of the soil to be examined is weighed (§ 75), put into the litre of sterilised water and shaken thoroughly for several minutes. One cubic centimetre is transferred from the 2-litre flask to the first test-tube, then 1 c.c from the first tube to the second and so on (§ 20). Each time, before pipetting off the 1 c.c., the tube is vigorously shaken. Dilutions representing $\frac{1}{10.000}$ to $\frac{1}{1.000.000}$ or $\frac{1}{100.000}$ to $\frac{1}{10.000.000}$ of a grm., according to the expected bacterial content of the soil, are transferred to Petri dishes labelled to correspond. The various media are added and carefully mixed with the 1 c.c. water. The plates are incubated and the colonies counted in the usual way (*see* §§ 17 and 34). The most frequently occurring germs, or say one to two types from each medium, are reinoculated, and pure cultures (§ 21) obtained by pouring fresh plates, in each case with the same kind of medium as before. Further examinations are made in the usual way (Ch. V.–VIII.).

Soil of average quality has usually a bacterial content, according to the results from plating, ranging between 100,000 and 10 millions, while in rich soil the numbers amount to from 1 to 100 millions per grm. For comparative experiments the samples must, of course, be taken in a similar manner. A hole is dug with a spade and the sides made smooth. With a clean spoon a sample is taken from the side, 8–12 cm. below the surface. Results should be confirmed by contro experiments. The experimental error in these experiments is very considerable, but this is no serious objection in the case of preliminary examinations.

Higher, but still more inaccurate, numbers may be obtained by inoculating from the above dilutions (which must be continued further), into tubes containing small quantities of the various special culture solutions described in the following chapters. At least four controls of each dilution are required to secure satisfactory results. To make the chemical and physical conditions as equal as possible, add some sterilised earth aseptically into the tubes before transferring the dilutions (*see* § 47).

108. **Catalase Test.** Soil, like milk (§ 53), decomposes hydrogen peroxide, but much more oxygen is liberated by the former than by the latter. The liberation of oxygen by soil is caused by humus, micro-organisms, and by inorganic soil particles. Usually the addition of 20 c.c. 3 per cent. hydrogen peroxide (1 part Merck's perhydrol + 9 parts dist. water) to 5 grms. soil is sufficient; but as much as 40 c.c. must be added to soil rich in humus to obtain complete evolution of oxygen. For comparative results it seems best to determine in what time 100 c.c. oxygen is liberated. The earth is put into a 300 c.c. Erlenmeyer flask, and mixed with 50 c.c. water. Then hydrogen peroxide is added, the mixture kept moving and the oxygen collected in a graduated tube over water. Experiments with the same earth after different treatment should be made. Test in this way (1) fresh earth; (2) the same earth when the germs have been killed and the catalase destroyed, by heating in the autoclave to 2 atmos.; (3) the same earth without humus, the humus being burnt off by heating strongly with the flame. The results from these three tests give a general idea of the part played by each factor.

XXXII. PRODUCTION OF CARBON DIOXIDE. INTENSITY OF FERMENTATION.

109. **Production of Carbon Dioxide.** The amount of gas evolved by the total germ life in soil may be determined in the same way as in dung. Also an approximate estimate of the decomposition of humus in soil may be obtained by collecting the carbon dioxide in special absorption apparatus (*see* § 95). In order to have the conditions as natural as possible the soil sample should be taken by driving a wide zinc cylinder into the soil. After removing the cylinder and making it air-tight, the production of carbon dioxide

should be studied while the soil is in its original position in the cylinder.

Reference: Hesselink van Suchtelen, *Centralbl. f. Bakt.*, II. Abt., **28**, p. 45.

110. **Intensity of Fermentation.** When an aqueous sugar solution (*e.g.* tap water + 4 per cent. glucose + 0·1 per cent. peptone + 0·05 per cent. K_2HPO_4) is infected with 10 per cent. earth, a large number of bacteria especially of the *Aerobacter* and *Amylobacter* groups produce an active evolution of carbon dioxide and hydrogen. By weighing the flask at the beginning and at the end of the experiment, the total loss of gas can be determined.

Reference: R. Albert and Luther, *Journal f. Landwirtschaft*, **56**, 1908, pp. 361 *et seq.*

XXXIII. AEROBIC PECTIN AND CELLULOSE DECOMPOSITION.[1]

111. **Aerobic Pectin Decomposition.** Ten cubic centimetres of the culture solution given in § 91 is put into each of three or four 50 c.c. Erlenmeyer flasks. One flask is infected with earth; after about eight days at 25° C. purification is obtained by progressive sub-culturing into the other flasks. For microscopic examinations Chinese-ink preparations are recommended. For isolating, flesh gelatine may be used. Pectin decomposition is brought about by various moulds, but especially by some spore-bearing bacteria (*B. asterosporus* and some varieties of *B. subtilis* and *mesentericus*). The most frequently occuring types should be re-inoculated and diagnosed.

112. **Aerobic Cellulose Decomposition.** Two filter papers are placed in each of two Petri dishes. In one of the dishes some $MgNH_4PO_4$ is spread between the two filter papers, which are moistened with a 0·05 per cent. aqueous solution of K_2HPO_4, some soil rich in humus added, and

[1] For anaerobic pectin and cellulose decompositions, which are also found in soil, *see* Chapter XXVI.

the plates incubated at 25°–30° C. In three to six days brown spots produced by bacteria appear.

In the other dish the paper is infected with the same earth, but moistened with the following solution : 100 tap water, 0·05 NH_4NO_3, 0·05 K_2HPO_4. Incubate, in this case, at room temperature, 18°–20° C., and moisten the paper from time to time. In two or three weeks various cellulose-decomposing moulds develop.

As growth proceeds detailed observations should be made under the microscope. So far, little more is known regarding pure cultures of the aerobic cellulose bacteria. The moulds above named can be isolated and examined in the usual way (*see also* § 86).

Regarding cellulose decomposition by denitrifying bacteria *see* § 122.

XXXIV. ASSIMILATION OF METHANE, HYDROGEN, AND CARBON MONOXIDE.[1]

113. **Assimilation of Methane.** The following solution (Söhngen), in shallow layers in Erlenmeyer flasks, may be used for relatively increasing the specific organisms : 100 dist. water, 0·1 $MgNH_4PO_4$, 0·05 K_2HPO_4, 0·01 $CaSO_4$.

The flasks are infected with manured soil, liquid manure, or irrigation water, and closed by two-holed stoppers provided with two glass tubes. Then an atmosphere of methane and oxygen is produced. Methane can be prepared from sodium acetate ; before allowing it to enter the flasks it should be washed with bromine water and caustic soda. After two to four days at 30°–37° C. an almost pure culture of *Bac. methanicus* is obtained as a red skin on the surface of the solution. It can be isolated on flesh gelatine.

References : Söhngen, *Centralbl. f. Bakt.*, II. Abt., **15**, p. 514 (with figures of specially constructed apparatus for this purpose) ; Kaserer, *Zeitschr. f. d. landw. Versuchs-Wesen in Oesterreich*, **8**, 1905, pp. 790 793 ; *Centralbl. f. Bakt.*, II. Abt., **15**, pp. 574 *et seq.*

[1] These experiments on the assimilation of methane and hydrogen may be combined with those on the production of these gases ; *see* Chapters XXVI and XXVII.

114. **Assimilation of Hydrogen.** The following solution may be used: 100 dist. water, 0·2 KNO_3, 0·1 $NaHCO_3$, 0·05 K_2HPO_4, 0·02 $MgSO_4$, trace of Fe_2Cl_6, in shallow layers in Erlenmeyer flasks (or in the air-tight fermentation apparatus of Hofstädter quoted on p. 44, the long arm of which is filled with oxy-hydrogen gas). After infection with earth an atmosphere of oxygen and hydrogen with some carbon dioxide is produced; the hydrogen is purified by being passed through $CuSO_4$, acid and alkaline $K_2Mn_2O_8$ and conc. H_2SO_4. Incubate at 27°–33° C. and isolate on flesh agar or on the agar referred to in § 115. On the former, chiefly short yellow rods are found (related to *Bact. turcosum*), on the latter *Bac. oligocarbophilus*.

References: Kaserer, *loc. cit.* (*see* § 113) and *Centralbl. f. Bakt.*, II. Abt., **16**, pp. 685 *et seq.* (*Bac. pantotrophus* is identical with *Bact. turcosum*); Nabokich and Lebedeff, *Centralbl. f. Bakt.*, II. Abt. **17**, p. 350; Niklewski, *Jahrb. f. wissenschaftl. Botanik*, **48**, 1910, pp. 113-142.

115. **Assimilation of Carbon Monoxide.** Owing to the presence of some carbon monoxide in the air of the laboratory from the burning gas the carbon-monoxide-assimilating *B. oligocarbophilus* often appears as a dry white skin on the surface of the solution in the flasks for the nitrification experiment (§ 100).

For the cultivation of this organism the same $(NH_4)_2SO_4$ solution is used, but without the addition of chalk. After infecting with earth, incubate at 20°–25° C. and from time to time allow some ordinary gas to enter the incubator. Isolating is done either on gypsum plates (*see* § 121) or on specially prepared agar. The agar is washed with distilled water till all soluble matter is removed, then 1½ per cent. of the following solution is added: 100 water, 0·01 KNO_3, 0·01 K_2HPO_4.

Reference: Beijerinck and van Delden, *Centralbl. f. Bakt.*, II. Abt., **10**, pp. 34 *et seq.*

XXXV. PRODUCTION OF AMMONIA FROM ORGANIC MANURES.

116. **Culture Media.** A small quantity of each of various organic manures in proportion to their nitrogen content (*e.g.* from meat meal, horn meal, or dried blood, 1 per cent.; from bone meal, dried green manure, &c., 3 per cent.) is weighed

and put into about 10 c.c. water in Erlenmeyer flasks or test tubes (in each case duplicates should be prepared, *see* §§ 117 and 118). Except where sufficient phosphate is already present in the material, as in bone meal, 0·05 per cent. K_2HPO_4 is also added. For comparison 1 per cent. peptone or gelatine can be used in the same way. The aerobic ammonia-producers are isolated on flesh gelatine, the anaerobes on flesh agar.[1]

117. **Aerobic Ammonia Production.** The Erlenmeyer flasks prepared above are infected with about 10 per cent. earth and one series of them are pasteurised for five minutes in the steriliser. After five to ten days at 20° C. a thick turbidity, surface growth, and more or less putrid odour are perceptible. The organisms present on the gelatine plates from the non-pasteurised flasks should be examined microscopically; chiefly short rods (*Bact. fluorescens, punctatum, vulgare, prodigiosum, coli, radiobacter*, etc.) and sometimes Cocci will be found, while from the pasteurised solution, as a rule, more vigorous ammonia producers, as *B. mycoides, subtilis, mesentericus*, etc., develop.

The activity of the pure culture can be tested in the respective solutions after adding some earth which has been heated in shallow layers to 200° C. in the air oven. The ammonia is distilled off by means of MgO.

By subtracting the amount of ammonia obtained from some sterile control flasks, the ammonia split off by the action of the bacteria can be estimated.

118. **Anaerobic Ammonia Production.** The tubes prepared as above are infected with earth. One series is pasteurised, anaerobic conditions produced, and the tubes incubated at 38° C. As soon as a vigorous fermentation begins (after about five days) examine microscopically, and isolate the specific anaerobes in the usual way (§ 41 *et seq.*). In the non-pasteurised tubes various streptococci pre-

[1] About 580 c.c. gelatine (for seventy tubes) and 320 c.c. agar (for forty tubes) are prepared from 1000 c.c. bouillon. The surplus bouillon is added to that already in stock (*see* § 106).

dominate, while in the pasteurised solution all forms of the group of *Bac. putrificus* are present, producing very disagreeable-smelling substances.

XXXVI. PRODUCTION OF AMMONIA FROM UREA AND CYANAMIDE.

119. **Urea Decomposition.** Besides the media given in § 97 the following albuminoid-free culture solutions are useful for the cultivation of urea bacteria from the soil.

1. Soil extract + 0·05 per cent K_2HPO_4 + 5 per cent. urea.
2. Söhngen's solution: 100 tap water, 5 urea, 0·05 K_2HPO_4, 0·5–1 ammonium or calcium malate, calcium citrate or tartrate.

Some test tubes are filled with each solution and, after infection with earth, are incubated at 20°–30° C. By pasteurising, spore-bearing bacteria can be selected (§ 117). Isolation may be accomplished on urea flesh gelatine or urea flesh agar (*see* § 97), or on agar prepared from the above solutions but with an addition of only 1½ per cent. urea. Besides *Bact. erythrogenes* and other yellow-growing short rods, varieties of *Bact. vulgare* are more commonly found; while *Bac. pumilus* and other spore bearers occur most frequently in the pasteurised solutions. *Bac. Pasteurii* is not found as it is only able to grow in the presence of albuminoids.

References: Löhnis, *Centralbl. f. Bakt.*, II. Abt., **14**, p. 714; Söhngen, *ibid.* **23**, pp. 94 *et seq.*

120. **Cyanamide Decomposition.** Add 0·2 per cent. calcium cyanamide, 0·01 per cent. asparagin, and 0·01 per cent. glucose to 150 c.c. of soil extract + 0·05 per cent. K_2HPO_4 (as prepared in § 106). Fifty cubic centimetres of this mixture is divided into some test tubes, one or two are at once infected with earth and the remainder are heated three times in the steriliser. Ten per cent. gelatine is added to the remaining 100 c.c.: the reaction in this case must be distinctly alkaline. After one or two

weeks, sub-cultures are made from the earth-infected solutions into the sterile solutions, and from these, later on, calcium cyanamide plate cultures are prepared. The organisms found are urea bacteria (mostly yellow-growing short rods) which convert the urea formed out of the cyanamide in the presence of soil colloids (humus, zeolites, etc.) into ammonia. They can be further investigated on the standard media.

Organisms which directly attack cyanamide are also present in the soil, but, as a rule, they do not play an important *rôle*. Germ-free cyanamide solutions obtained by filtration through porcelain (§ 1, p. 8) generally remain unaltered after infection with soil extract rich in bacteria. It is only in presence of colloid substances that an active urea formation, followed by ammonia production, takes place. Heating the calcium cyanamide solution in steam, like the addition of earth, makes the development of these bacteria possible. If the solution is prepared with free cyanamide instead of calcium cyanamide and made slightly acid (with some drops of lactic acid) it serves for the cultivation of different moulds which are able to attack the cyanamide directly.

The intensity with which cyanamide decomposition takes place in different soils can be tested by mixing the respective samples of soil with suitable amounts of calcium cyanamide and making germination experiments after a shorter or longer time. Incomplete decomposition of the poisonous cyanamide is indicated by disturbances in the germination.

References: Löhnis, *Centralbl. f. Bakt.*, II. Abt., **14**, pp. 87, 389; Ulpiani, *Gazetta chimica ital.*, **40**, 1910, p. 613 (ref. *Centralbl. f. Bakt.*, II. Abt., **29**, p. 235); Reis, *Biochem. Zeitschrift*, **25**, pp. 460, 477; Kappen, *Centralbl. f. Bakt.*, II. Abt., **26**, p. 633. Regarding cyanamide preparation and cyanamide and urea estimation *see* Caro, *Zeitschr. f. angewandte Chemie*, **23**, 1910, pp. 2405 *et seq.*

XXXVII. NITRIFICATION. DENITRIFICATION. NITRATE REDUCTION.

121. **Nitrification.** Nitrifying bacteria do not grow on the ordinary solid media. Pure cultures may be obtained

by the methods of cultivation[1] described in § 100 either in conjunction with the Chinese-ink point method (§ 48) or by using special media (at 25°–30° C.). Many different methods have already been tried, but the obtaining of pure cultures of the nitrifying organisms is still a most difficult bacteriological problem. The method which is most to be recommended for the cultivation of the **nitrate bacteria** is the gypsum-magnesia-plating method proposed by Omelianski and Makrinoff:

300 grms. gypsum, 3 grms. $MgCO_3$, and 3 grms. $MgNH_4PO_4$ are carefully mixed together and moistened with an unfiltered water extract of soil rich in humus (about 250 grms. of earth per litre of tap water) and the whole stirred into a pasty mass. The mass is put on a glass plate and spread out so that it has a height of about $\frac{1}{2}$–$\frac{3}{4}$ cm. From this are cut out (with a glass dish about 8 cm. in diameter) suitable slices for Petri dishes, and (with a knife) suitable strips to go into test tubes. When thoroughly hardened these preparations are sterilised in the air oven. They are moistened from below with the culture solution which, in this case, is prepared without ammonium sulphate and basic magnesium carbonate. The surface is inoculated from the culture solution and the cultures kept at 25°–30° C. Besides the yellow-brown nitrite bacteria, white colonies of *Bac. oligocarbophilus* develop (*see* § 115).

For nitrate producers Winogradsky used the following nitrite agar:

1000 water, 15 agar, 2 $NaNO_2$, 1 Na_2CO_3, 0·5 K_2HPO_4.

While the nitrite bacteria can be stained in the ordinary way, the spore method (§ 36) is best adapted for the nitrate organisms.

References: Winogradsky, *Centralbl. f. Bakt.*, II. Abt., **2**, p. 425; Omelianski, *ibid.*, **5**, p. 652; Makrinoff, *ibid.*, **24**, p. 419.

122. **Denitrification.** Besides the solutions already given in § 101 (nitrate houillon and Giltay's solution) the

[1] Very active nitrification is usually got in soil extract solution + 1 per cent. $(NH_4)_2SO_4$ + 0·5 per cent. K_2HPO_4 + chalk after infection with earth.

following culture solutions can be specially used for the denitrifying bacteria in soil:

(*a*) 100 tap water, 2 calcium tartrate, citrate or malate, 0·1–2 KNO_3, 0·05 K_2HPO_4.
(*b*) 100 tap water, 2 filter paper or cotton-wool (cellulose), 0·25 KNO_3, 0·05 K_2HPO_4.

After inoculating with earth, incubate at 38° C., and when vigorous fermentation is seen, make sub-cultures in the same solution, repeating several times. The specific bacteria (*Bact. Stutzeri, fluorescens*, etc.) are isolated on nitrate agar (*see* § 101) and further cultivated on the standard media.

Reference: Van Iterson, *Centralbl. f. Bakt.*, II. Abt., **11**, pp. 690 *et seq.*, **12**, pp. 108 *et seq.*

123. **Nitrate Reduction.** As in dung (§ 102), many types of nitrate reducing bacteria can readily be found in soil. The cultures of soil organisms already in hand may be examined according to the method given in § 102.

XXXVIII. SYMBIOTIC NITROGEN-FIXING BACTERIA.

124. **Microscopic Examination of the Nodules on Leguminous Plants.** Wash some nodules from the roots of different Leguminosæ, cut them aseptically, and make stained smear preparations from them. Study the variation in the shape of the bacteria and their different reactions to Gram's stain (§ 35). With a microtome cut sections of the nodules and examine them carefully.

The slimy mass containing the bacteria, which fills young nodules, can be specially well examined by Hiltner's method (Lafar's *Handbuch d. techn. Mykologie*, Bd. 3, p. 40). The sections which have been cut are put in a stain bath containing equal parts of methylene blue and fuchsin dissolved in 1 per cent. acetic acid. The cells of the nodule are stained blue, the bacteria red, while the slime threads are not stained at all.

125. **Isolating the Nodule Bacteria.** One per cent. mannite is added to 250 c.c. soil extract + 0·05 per cent. K_2HPO_4 (prepared as in § 106). 125 c.c. of this solution is mixed with 1½ per cent. agar. The agar is filled into test tubes, and the mannite soil-extract partly into small Erlenmeyer flasks and partly into test tubes. The tubes and flasks are sterilised in the autoclave or in the steamer.[1]

Some well-developed young nodules are dipped first for five minutes into a 1 per cent. solution of corrosive sublimate, then into alcohol. The last traces of alcohol are removed by passing the nodule quickly through the flame. The nodule is then placed on a sterilised surface (heated slide, etc.) and cut open with a sterilised knife. The inner part of the nodule is inoculated into a mannite agar tube which has been previously melted and cooled to 45° C. The required dilutions are made in the same medium and plates poured. Plates can also be prepared from the commercial bacterial preparations (Azotogen, Nitragin, etc.). The colonies develop after a few days, those of the nodule bacteria generally predominating. These have the appearance of round, white, translucent, slime-like drops, finely granulated and often with compact white centres. They contain the normal, short, rod forms, which during the first days are very motile. Mannite agar is the most suitable medium for further cultivation. A very meagre growth, or perhaps none at all, is obtained on the standard media.

Sometimes the roots of leguminous plants show, instead of the normal nodules, crown-gall tumours caused by *B. tumefaciens* which somewhat resembles *B. radicicola*. For a rapid diagnosis the following medium is recommended: 100 parts water, 1 sugar, 0·1 K_2HPO_4, 0·02 $MgSO_4$, 1·5 agar, 0·01 Congo red. *B. radicicola* forms white colonies, while *B. tumefaciens* absorbs the Congo red and therefore produces red or reddish colonies. For further information see K. F. Kellerman, U.S. Dep. of Agriculture, Bureau of Plant Industry, No. 76, March 1911.

[1] If desired, the surplus bouillon (note 1, p. 107) can at the same time be utilised for making gelatine agar and glucose agar.

126. **Nitrogen Fixation and Infection Experiments.** If pure cultures are inoculated into mannite soil-extract, in eight to fourteen days a considerable amount of white slime is formed in which, after a sufficiently long time, the typical branched and swollen forms (as inside the nodules) are obtained. If a sufficient amount of solution is used for the experiment (100 c.c. in 300 c.c. Erlenmeyer flasks) a distinct increase in the nitrogen content can be detected after three weeks (Kjeldahl). The power of fixing nitrogen, which an old culture may have lost, can be restored by inoculating the bacteria from such cultures into sterilised earth in test tubes.

The vitality of a culture may be tested (Harrison and Barlow) by putting aseptically-grown seedlings [1] of the plants being tested into a 2-litre flask containing ash-maltose agar (4 grms. wood ashes extracted with 1000 c.c. tap water and 4 per cent. maltose + 2 per cent. K_2HPO_4 added to the extract). The plants can be easily kept sterile in the vessels closed with cotton-wool, and the effects of any inoculation are easily seen in the transparent medium.

Reference: Harrison and Barlow, *Centralbl. f. Bakt.*, II. Abt., **19**, p. 268 (photograms are given).

XXXIX. FREE-LIVING, NITROGEN-FIXING BACTERIA.

127. **Aerobic, Nitrogen-Fixing Bacteria.** Mannite soil-extract in small Erlenmeyer flasks (*see* § 125) is infected with earth and kept for five to ten days at 20°–30° C. A wrinkled skin, white at first, brown later, is formed (more certain in winter than in summer, and more regularly by using rich soil than poor or sour soil). An illustration of a stained specimen is shown in Fig. 39. Single, small,

[1] The seeds are thoroughly cleaned with water, put for thirty minutes into a 2 per cent. solution of corrosive sublimate, and then carefully washed with sterilised water. By laying the seeds on sterile gelatine plates in Petri dishes the absence of bacteria and moulds can be tested.

thin bacilli are visible between the large cells of *Azotobacter*. The former are almost always a type resembling the nodule bacteria called *Bact. radiobacter*, and can also fix nitrogen to a slight extent. Besides these, especially when *Azotobacter* is less in evidence, many other sporing and non-sporing bacteria participate in the process. *Azotobacter*, however, is the most vigorous, free, nitrogen-fixing organism yet discovered.

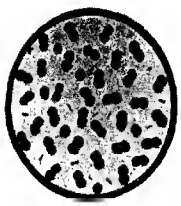


FIG. 39. Azotobacter. Mixed culture (× 700 diams.)

Its separation from *radiobacter* is, on account of the slimy property of its cell wall, often very difficult. The quickest way is to re-inoculate first into mannite soil-extract.

On the standard media *Azotobacter* grows only moderately, but, on the contrary, very well on gypsum plates which have been wetted with mannite solution. Those varieties which become brown or black yield specially characteristic pictures on this medium. The amount of nitrogen fixed by pure cultures in mannite solution is often considerably increased by adding humus, or soil rich in humus, especially if iron salts are present.

References: Beijerinck, *Centralbl. f. Bakt.*, II. Abt., **7**, p. 574 (*Azotobacter* photogram); Löhnis, *idem*, **14**, pp. 582 *et seq.* (*Bact. radiobacter* and other nitrogen fixers); Löhnis and Westermann, *ibid.* **22**, pp. 234 *et seq.* (comparative experiments with different types of *Azotobacter*); Krzemieniewski, *Anzeiger d. Akad. Krakau, mathem.-naturw. Klasse*, 1908, pp. 993 *et seq.* (Test of Action of Humus); Remy and Rotrug, *Centralbl. f. Bakt.*, II. Abt., **30**, 1911, pp. 369-384.

128. **Anaerobic, Nitrogen-Fixing Bacteria.** When *Azotobacter* is not abundant, the mannite solution frequently produces vigorous butyric fermentation. In this case the active forms of the group *Bac. amylobacter* are certain to

appear if 10 c.c. of an aqueous, dextrose solution (2 grms. dextrose in 100 c.c. water) in test-tubes is richly infected with soil, pasteurised for fifteen minutes at 80° C., and incubated at 30°–38° C. Isolation can be accomplished on glucose flesh agar in Burri tubes (§ 41) and further investigation made under anaerobic conditions on media containing sugar. Pasteurisation must be repeated from time to time. All the forms of *Amylobacter*, which, for example, have been reared on milk, flax, or other media, can take the property of fixing nitrogen when cultivated in sterilised soil.

129. **Nitrogen-Fixing Algæ.** A more or less vigorous vegetation consisting chiefly of blue-green algæ can be obtained by infecting tap water + 0·02 per cent. K_2HPO_4 with 1-2 per cent. earth and keeping it for four to eight weeks well-aerated and in the light. Among these algæ some nitrogen-fixing sorts occur which may be isolated, but with difficulty, on agar which has been carefully washed and soaked with an aqueous solution of potassium phosphate. In symbiosis with the algæ, nitrogen-fixing bacteria are active, which receive from the algæ the carbon compounds they require and give back in exchange nitrogenous food. If such an experiment is continued for months and years there results gradually, as in nature on moist, sandy places poor in nitrogen, a thick, green layer of algæ, the nitrogen-content of which is taken almost entirely from the air.

XL. CHANGES IN MINERAL SUBSTANCES.

130. **Phosphate Solution.** Tap water + 2 per cent. glucose is filled into small Erlenmeyer flasks, to which a little earth and some di- or tri-calcic phosphate have been previously added. Incubation is at 38° C. and, after the fermentation has continued for some days, plates are poured with soil extract agar + 2 per cent. glucose. Before the plates are poured a little di- or tricalcic phosphate, which has been previously sterilised in test-tubes in the air oven, is put into each dish. The phosphate is mixed uniformly with the agar. The acid-producing colonies

which develop at 38° C. dissolve the phosphate and become surrounded by a clear area, similar to that produced by lactic acid bacteria on chalk agar (Plate III., Fig. 1).

131. **Iron Bacteria.** The presence of iron bacteria in water can be detected by adding to the water 0·05 per cent. ferric-ammonium citrate and keeping it in well-filled test tubes. A yellowish skin, flakes, or a precipitate of iron bacteria, according to circumstances, is obtained after some time. The solution should be tested microscopically. As culture medium H. Molisch recommended a gelatine made from peat extract (peat extracted with boiling water) + 0·25 per cent. manganese-peptone.

Reference: Molisch, *Die Eisenbakterien.* Jena, 1910.

132. **Production of Sulphuretted Hydrogen.** That many types of bacteria produce sulphuretted hydrogen in flesh bouillon can be shown by hanging a piece of lead acetate paper in the tubes (*see* § 31). Vigorous evolution of this gas takes place when some flowers of sulphur are added to bouillon infected with earth, dung, etc. For rapid detection of the specific bacteria the white lead test (Beijerinck) can be used. The infected flesh agar, or flesh gelatine, is mixed with lead carbonate in the Petri dish. The H_2S-producing colonies become surrounded with a brown circle. A further test is that by Fromme, in which the flesh gelatine is mixed with 3 per cent. ferric tartrate, which is blackened by H_2S.

References: Beijerinck, *Centralbl. f. Bakt.*, II. Abt., **6**, p. 196; Fromme, *Über die Beziehungen des metallischen Eisens, u.s.w.*, Diss. med. Marburg, 1891.

APPENDIX.

APPENDIX.

I. TABLE FOR THE IDENTIFICATION OF BACTERIA.

Micrococcus, Cohn.

Mostly round cells, single or grouped irregularly, never in chains or in packets.

A.—White, grey, yellow or orange on gelatine and agar.

I. Gelatine liquefied, milk coagulated.

(*a*) White—(*α*) Chiefly pathogenic form.
M. pyogenes γ albus (Rosenb.), Lehm. et Neum.
(*β*) Non-pathogenic form.
M. acidi lactis, R. Krüger.

(*b*) Yellow—(*α*) Chiefly pathogenic form.
M. pyogenes β citreus (Passet), Lehm. et Neum.
(*β*) Non-pathogenic form.
M. luteus, Cohn, emend. Lehm. et Neum.

(*c*) Orange—(*α*) Chiefly pathogenic form.
M. pyogenes α aureus (Rosenb.), Lehm. et Neum.

II. Gelatine not liquefied, milk coagulated.

(*a*) White . . . . . *M. lactis acidi*, Leichm.
(*b*) Yellow . . . . . *M. lactis acidi*, Marpm.
(*c*) Orange—reddish brown. . *M. umbilicatus*, R. Weiss.

III. Gelatine liquefied, milk not coagulated.

(*a*) White . . . . . *M. butyri*, Keith.
(*b*) Yellow . . . . . *M. chromoflavus*, Huss.
(*c*) Greyish orange. . . *M. cremoides*, Zimmerm.
(*d*) Brownish yellow . . *M. badius*, Lehm. et Neum.

IV. Gelatine not liquefied, milk not coagulated.

(*a*) White—(*α*) Non-motile. . *M. candicans*, Flügge.
(*β*) Motile . *M. agilis albus*, Catterina.
(*b*) Yellow . . . . . *M. sulphureus*, Zimmerm.
(*c*) Orange . . . . . *M. aurantiacus*, Cohn.

(*d*) Brownish yellow . *M. versicolor* (Guillebeau), Lhs.
(*e*) Translucent, iridescent, surface growth.
M. concentricus, Zimmerm.

V. Slime production, especially in milk.
(*a*) Gelatine liquefied . *M. Freudenreichii*, Guillebeau.
(*b*) Gelatine not liquefied. *M. pituitoparus* (Hohl), Lhs.

B.—Red to cherry-red on gelatine and agar.
(*α*) Non-motile. *M. roseus* (Bumm), Lehm. et Neum.
(*β*) Motile . . . . . *M. agilis*, Ali-Cohen.

C.—Cobalt-blue on gelatine and agar.
M. cyaneus (Schröter), Cohn.

Sarcina, Goodsir.

Mostly round cells; when cultivated in liquid media, in packet orm.

A.—White, grey, yellow or orange on gelatine and agar.
(*a*) White-grey. (*α*) Non-motile . *S. alba*, Zimmerm.
(*β*) Motile, spore-bearing.
S. pulmonum, Virchow.
(*b*) Yellow. (*α*) Non-motile.
S. lutea, Flügge, emend. Lehm. et Stubenr.
(*β*) Motile, spore-bearing
S. ureae (Beijerinck), Lhs.
(*c*) Orange . . . . . *S. aurantiaca*, Flügge.
(*d*) Brownish yellow . . . *S. fulva*, Stubenrath.

B.—Red to cherry-red on gelatine and agar.
S. rosea, Schröter, emend. Zimmerm.

Streptococcus, Billroth.

Round, pointed, sometimes distinctly short, rod-shaped cells, in chains, especially when cultivated in liquid media.

A.—Gelatine not liquefied.

I. Milk coagulated.
(*a*) Gas production from glucose.
Str. mastitidis, Guillebeau.
(*b*) No gas production from glucose.
(*α*) Cells round or disc-shaped; often pathogenic.
Str. pyogenes, Rosenb.

(β) Cells round, pointed, or (especially in bouillon) short, but distinctly rod-shaped.

Str. lactis (Lister), Lhs.

II. Milk not coagulated.

(*a*) Gas production from glucose. . *Str. Kefir*, Migula.

(*b*) No gas production from glucose.

Str. lactis innocuus, Lhs.

III. Slime production in milk. *Str. hollandicus*, Scholl.

B.—Gelatine liquefied.

(*a*) White growth. *Str. gracilis* (Escherich), Lehm. et Neum.

(*b*) Yellow to greenish yellow.

Str. coli brevis, Escherich.

Bacterium, Cohn, emend. Hueppe.

Non-sporulating rods, generally comparatively short, sometimes in threads, straight or (seldom) slightly curved.

A.—Do not grow, or only very poorly, on flesh media.

1. Long, non-motile rods, often distinctly anaerobic.

Bact. caucasicum (v. Freudenr.), Lehm. et Neum.

2. Short, sometimes motile rods; distinctly aerobic.

Bact. radicicola (Beijck.), Prazm.

B.—Grow well on flesh media.

I. White to grey, sometimes faintly reddish. Media generally not coloured. Agar and gelatine seldom brownish.

1. Round, crenate or leaf-like colonies.

(*a*) Gelatine not liquefied.

(α) Non-motile.

1. Milk coagulated, gas production from milk sugar.

Bact. acidi lactici (Hueppe).

Bact. aerogenes (Escherich), Lehm. et Neum.

2. Milk not coagulated. Gas production from milk-sugar . . *Bact. pneumoniae*, Friedldr.

3. Milk coagulated. No gas production from milk-sugar . . *Bact. limbatum*, Marpmann.

4. Milk not coagulated. No gas production from milk-sugar.

Bact. lactis innocuum (Wilde), Lhs.

5. Vigorous slime production in milk.

Bact. lactis viscosum (Adametz), Lehm. et Neum.

(β) Motile.

1. Milk coagulated. Gas production from milk-sugar.
Bact. coli (Escherich), Lehm. et Neum.
2. Milk not coagulated or slowly (by rennet). No gas production from milk-sugar.
Bact. radiobacter (Beijck.), Lhs.
3. Milk not coagulated. No gas production from milk-sugar. Tough-skinned, dry, very wrinkled colonies. Denitrifying.
Bact. Stutzeri, Lehm. et Neum.
4. Milk not coagulated. No gas production from milk-sugar. Characteristic crumbly colonies with curved out-growths. The cells show distinct polar staining; involution forms are common. Vigorous slime production in nitrate solution.
Bact. agreste, Lhs.

(b) Gelatine liquefied.

(a) Non-motile.
Bact. disciformans (Zimmerm.), Lehm. et Neum.

(β) Motile.
Bact. punctatum (Zimmerm.), Lehm. et Neum.

2. Irregular colonies with vermicular, ligamentous, rhizoid or radiating out-growths, sometimes surrounded by very small, spot-like colonies.
Bact. vulgare (Hauser), Lehm. et Neum.

II. White-grey. Media coloured green, blue, brown, or black.

1. Gelatine liquefied, media generally green, seldom brown.
Bact. fluorescens (Flügge), Lehm. et Neum.
2. Gelatine not liquefied.

(a) Media generally green, seldom brown.
Bact. putidum (Flügge), Lehm. et Neum.

(b) Media blue to black.
Bact. syncyaneum (Ehrenbg.), Lehm. et Neum.

III. Yellow (sometimes greyish, or greenish yellow) to orange.

1. Intensive greenish yellow; very small, actively motile rods, gelatine slowly liquefied (napiform).
Bact. turcosum (Zimmerm.), Lehm. et Neum.
2. Bright lemon to gold yellow.

(a) Non-liquefying, non-motile.
Bact. luteum (Flügge), Lehm. et Neum.

(*b*) Liquefying, non-motile.

Bact. helvolum (Zimmerm.), Lehm. et Neum.

(*c*) Liquefying, motile.

Bact. herbicola, Burri et Düggeli.

3. Lemon yellow. Media coloured red.

Bact. erythrogenes (Grotenf.), Lehm. et Neum.

4. Yellowish white to egg yellow or greyish orange, seldom changing to brownish yellow.

(*a*) Non-liquefying, non-motile.

Bact. cremoides, Lehm. et Neum.

(*b*) Liquefying, non-motile.

Bact. nubilum, Lehm. et Neum.

(*c*) Liquefying, motile.

Bact. ochraceum (Zimmerm.), Lehm. et Neum.

5. Yellow, orange to brick red.

(*a*) Non-motile. *Bact. fulvum* (Zimmerm.), Lehm. et Neum.

(*b*) Motile . . . . *Bact. chrysogloea*, Zopf.

IV. Rose red, carmine, rust-red to brownish red.

(*a*) Non-liquefying, non-motile.

Bact. latericium (Adametz), Lehm. et Neum.

(*b*) Non-liquefying, motile. *Bact. Coccardi* (Huss), Lhs.

(*c*) Liquefying; motile.

Bact. prodigiosum (Ehrenbg.), Lehm. et Neum.

V. Blue to violet.

1. Violet . . *Bact. violaceum* (Schröter), Lehm. et Neum.

2. Indigo blue.

Bact. indigonaceum (Claessen), Lehm. et Neum.

3. Sky blue to blueish green.

Bact. caeruleum (Voges), Lehm. et Neum.

Bacillus, Cohn, emend. Hueppe.

Sporulating, straight rods, often of considerable size.

A.—Do not grow, or only very poorly, on flesh media.

I. Aerobic. Decompose Urea.

B. Pasteurii (Miquel), Migula.

II. Anaerobic. Decompose cellulose.

1. Produce Hydrogen.

B. fermentationis cellulosae, Omelianski.

2. Produce Methane. *B. methanigenes*, Lehm. et Neum.

B.—Generally grow well on flesh media under aerobic conditions.

I. Gelatine stab with thorn-like projections (villous).

1. Tender, root-like projections; slow motility.
B. mycoides, Flügge.
2. Hairy projections, cloud-like, sticking together; actively motile. *B. sphaericus*, A. Meyer et Neide.
3. Projections very short; non-motile.
B. Ellenbachensis, Caron.

II. Gelatine stab without projections.

1. Thin translucent culture on potato.
B. tumescens, Zopf.
2. Pure white, convex culture on potato. *B. oxalaticus*, Zopf.
3. Greyish white, shallow, dusty culture on potato.
B. subtilis, Cohn.
4. Cream-coloured to reddish, slimy culture on potato (like *Bact. pneumoniae*). *B. pumilus*, A. M. et Gottheil.
5. Yellow-brown culture on potato (similar to *Bact. coli*).
(*a*) Slow motility.
(*α*) Like *subtilis* on gelatine and agar.
B. megaterium, De Bary.
(*β*) Yellow-coloured, agar becomes brown, napiform liquefaction of gelatine.
B. petasites, A. M. et Gottheil.
(*b*) Quick motility.
(*α*) Grey, translucent on gelatine and agar; very large spores. *B. asterosporus* (A. M.), Migula.
(*β*) Yellow, short rods with cylindrical spores.
B. parvus, A. M. et Neide.
(*γ*) Yellow to dark reddish brown. Agar brownish black, elliptical spores.
B. silvaticus, A. M. et Neide.
6. Potato culture shows folded surface elevation.
(*a*) Greyish-white. *B. mesentericus vulgatus*, Flügge.
(*b*) Yellowish. *B. mesentericus fuscus*, Flügge.
(*c*) Rose red, reddish brown.
B. mesentericus ruber, Globig.
(*d*) Grey blue, brown black.
B. mesentericus niger, Lunt.

C.—Obligate anaerobes. Grow well on flesh media.

1. Vigorous decomposition of albuminoid; no production of butyric acid. Rods generally drum-stick shaped during sporulation (*Plectridium*). *B. putrificus*, Bienstock.
2. Albuminoid decomposition and butyric acid production. Rods generally drum-stick shaped during sporulation (*Plectridium*). *B. paraputrificus*, Bienstock.
3. Butyric acid production, no decomposition of albuminoid. Rods generally club shaped during sporulation (*Clostridium*).

   B. amylobacter, van Tieghem emend. A. M. et Bredemann

   (*a*) Motile. *B. amylobacter var. mobilis.*

   (*b*) Non-motile. *B. amylobacter var. immobilis.*
4. Long, thin, bristle or pin-shaped rods; living on the products of *B. putrificus*. *B. postumus*, Heim.

II. LECTURE DEMONSTRATIONS.

THE **work**, not the shape, of micro-organisms, is what the agriculturist wants to know. Indeed, the form of a bacterium is of very little value for diagnosis; also, the expense of a good microscope is considerable and there is no small risk in placing such an instrument in the hands of inexperienced students. For these reasons, a microscope can be entirely dispensed with at demonstrations. A few good diagrams serve the purpose adequately, or the three main forms assumed by bacteria may be illustrated by a billiard ball, a lamp cylinder, and a corkscrew.

For lecture experiments it is preferable to use larger vessels than usual. Instead of the usual Petri dishes of 10 cm. diameter, larger ones with a diameter of about 12–15 cm. should be used; instead of 50 cm. Erlenmeyer flasks, 300–500 cm. flasks, or round ones of 1-2 litres capacity, serve better. Test tubes should be chosen of about 25 × 220 mm. fitting into demonstration cases (*see* App. III.).

Dissemination, Growth, and Properties of Bacteria. The germ content of air, water, milk, butter, cheese, dung, and soil may be shown according to §§ 16, 20, 50, 75, 80, 89, and 107.

Pure cultures of the more common bacteria (white and yellow micrococci from air and milk, *B. fluorescens*, hay and potato

bacilli, etc.), which have been either isolated from the plate cultures or obtained directly from some bacteriological laboratory, may be inoculated on the standard media (§§ 29–31). Good cultures can be preserved according to the directions given in Appendix III.

The **hay bacteria** are specially suited for demonstrating the heat-resisting powers of spores (*see* § 33), while the **butyric acid bacteria** from decomposing potatoes are examples of organisms which develop in absence of air (§§ 40, 41).

With regard to the **enriching** methods, which are, in their various aspects, of great importance in agricultural bacteriological work see § 46.

Dairy Bacteriology. The primary methods in dairy bacteriology (namely plating, sedimentation, reduction, catalase, boiling, alcohol, and fermentation tests) have been dealt with in §§ 50–56. Of special interest is the method for the examination of milk adopted by the Deutsche Landwirtschafts-Gesellschaft (*see* ref.[1] in § 56). If the catalase test is demonstrated with the same milk after it has been kept for some time, the gradual **increase** of bacteria can be distinctly shown.

The information about **foreign infection** (§ 57) is of special practical importance.

The activity of **acid-producing** and **casein-dissolving** bacteria is well shown by the methods given in §§ 60 and 63. **Slime-producing** bacteria can be increased as in § 66.

The abnormal bacterial condition of **heated** milk, and also the great difficulty of making milk absolutely sterile, is dealt with in §§ 71–73.

Dung and Soil Bacteriology. Experiments showing the **evolution of CO_2** can be very instructively combined with the plating methods (§§ 89 and 107). The vigorous gas development from dung is shown by simply keeping some dung in a stoppered flask. The various aspects of **pectin and cellulose decomposition** are dealt with in §§ 91, 92, 111 and 112.

Good demonstrations can be given on the **production of ammonia** from organic manures, urea, and cyanamide according to §§ 97, 117, 118, 120. **Nitrification** experiments (§ 100) are of special interest, since, without any external indication, very active chemical change may be taking place. Experiments to show **denitrification** (§ 101) should be carried out in deep and shallow solutions (the same solution in test tube and in Erlenmeyer flask) to demonstrate that active denitrification takes place only when air is pretty well

excluded, and that, therefore, in well-aerated, arable land little or no loss of nitrogen can be caused in this way.

Pure cultures of the **nodule bacteria** are comparatively easily made, as in § 125. The roots of lupins are usually well adapted for showing the bacterial tenants of the nodules. With regard to infection experiments, *see* § 126. *Azotobacter* (§ 127) can be relatively increased, not only on mannite solutions infected with earth, but also on gypsum plates with a little earth spread on the surface and moistened with mannite solution. By examining different types of soil (clay, sandy, peaty, etc.) at the same time, very different pictures are obtained.

Dicalcium phosphate plates (§ 130) are specially suitable for showing the **dissolving of phosphates,** while the white lead test shows the **production of sulphuretted hydrogen** by bacteria.

By infecting with the same earth the different culture solutions in shallow and in deep layers simultaneously, and cultivating at different temperatures, it may be demonstrated, first, that quite different groups of bacteria are present together in the soil; secondly, that there are bacteria, side by side, producing exactly opposite results; and, thirdly, that it depends entirely upon the physical and chemical conditions (cultivation, manuring, and cropping of the soil) what groups of bacteria become active.

III. PERMANENT PREPARATIONS.

Freshly prepared cultures of bacteria are generally best, but permanent preparations are also often very desirable. In many cases a bacteriological collection is almost indispensable. Specimens may be purchased from certain bacteriological laboratories, but they are naturally somewhat expensive.

Good permanent preparations are obtained by the following methods: Plate cultures are preserved by placing the under half of the Petri dish, with the medium upside down, on a sterilised glass plate and making it air-tight by means of plasticine. Several plates may be placed side by side on one glass pane and covered with a wooden case provided with a glass lid. With gelatine plates, it is generally necessary to kill the colonies, specially when liquefying bacteria are present. For this purpose a piece of filter paper is placed in the lid of the Petri dish (turned upside

down) and moistened with formalin. The vapour of the formaldehyde not only kills the colonies but generally causes the liquefied gelatine to solidify again (but not in every case), and at the same time the appearance of the colonies is unaltered (*i.e.* they still appear as if liquefying). Unfortunately, colours are destroyed, and the appearance of the plates spoiled, by excessive drying, especially if the formalin is allowed to act too long. Hence so far as possible, especially with agar plates, it is preferable simply to make the plates air-tight, and to do this early while the colonies are still young.

Very pretty and lasting preparations of **giant** colonies for demonstration purposes may be kept in Soyka flasks (§ 28) if the end of the cotton-wool plug is burnt in the flame, the plug pushed into the neck of the flask, and melted pitch poured on to the plug to close the flask air-tight. The pitch is melted by heating in a porcelain dish.[1] Several of these flasks can be mounted side by side by putting the necks into corresponding holes of a rectangular wooden block. The flasks are fixed by plugging the holes with cotton wool, and labels are put on the front of the case, preferably in such a way that they can be easily changed.

The preservation of test-tube cultures is done in a similar manner. In the case of liquefying cultures moistening of the cotton-wool plug with formalin should not be omitted. The best results are obtained by timely closing with pitch or plasticene. Exhibition cases, each for six tubes, are specially recommended; they are similar in construction to test-tube stands; but at the back and in front glass sides are placed, and the top is closed by a wooden lid. Labels are placed in corresponding slots on one side.

Good lasting preparations can be made from milk, and especially from the **fermentation tests**, by adding a little formalin and keeping in air-tight vessels. The curd cylinders should, however, be first hardened by placing them in absolute alcohol for several weeks. Very good sections can then be prepared, and kept in alcohol or diluted formalin.

[1] Spots of pitch are readily removed by means of xylol. Plasticine is also soluble in xylol.

IV. LABORATORY EQUIPMENT.

The more important **apparatus and instruments** have been described in Chapter I., and it has already been stated that, with the exercise of a little ingenuity, simple contrivances may be easily constructed which answer the purpose often quite satisfactorily. With regard to larger flasks, gypsum plates, etc., for demonstration purposes, see Appendix II. A supply of rubber tubing, thermometers, thermoregulators, porcelain dishes, and other laboratory essentials will be required, according to the nature and amount of work which is to be undertaken. For most purposes, and especially for weighing the ingredients of culture solutions, quite a simple balance is sufficient (*e.g.* the small balance with horn scales used in dispensaries).

The windows of the **laboratory** should, if possible, face the north. A draught chamber for the steam steriliser is desirable, and an ice chest for the summer months is also often very useful. Besides the microscope table, a special bench (for boiling, etc.) should be available, and also a drying stand.

There should be no drawers below the **microscope table** for a length of about 3–4 feet (*see* Fig. 8 on p. 11). The drawers for apparatus and a box for the microscope case, etc., may be situated on either side at right angles to the bench in order to save space. The bench should have a breadth of about two feet and the distance from the window should not exceed three feet. Simple three-legged **stools** are quite suitable for the bacteriological laboratory.

To keep glass apparatus, etc., free from dust, a glass **case** is desirable. Suitable sets of **shelves** on the wall are necessary for chemicals, pots, test-tube stands, etc. (Breadth 20–30 cm.; distance between shelves according to the purpose for which they are used, 20–32 cm.).

The following wood-stain (recommended by Wortmann) has proved particularly suitable for tables and shelves:

Solution I.	Solution II.
100 grms. copper sulphate.	100 grms. anilin chlorhydrate.
50 grms. potassium chlorate.	40 grms. ammonium chloride.
615 c.c. water.	615 c.c. water.

The solutions are applied alternately three times between each application the wood must be allowed to dry. Thereafter the

green scum is removed with warm water and, when dry, a little boiled linseed oil is rubbed on with a soft cloth, and the wood finally washed with soap and water. When treated in this way the wood takes on a uniform black colour and is very resistant to acid and alkali.

Illustrated descriptions of large bacteriological laboratories are found in the following works:

Aderhold, Die Kaiserl. Biologische Anstalt für Land- und Forstwirtschaft in Dahlem, *Dtsche. landw. Presse*, 1905, p. 673 und *Mitt. a. d. Biolog. Reichsanstalt, Heft.* 1, 1906;

v. Freudenreich, Das bakteriologische Laboratorium der schweizerischen landw. Versuchsanstalten Leibefeld-Bern, *Centralbl. f. Bakt.*, II. *Abt.* **13**, p. 163;

Knoll und Kornauth, Die K. K. landw.- bakteriologische und Pflanzenschutz-Station in Wien, *Zeitschr. f. d. landw. Vers.-Wesen in Oesterreich*, 1902, p. 697;

L. Russell, Description of the Laboratories of Agricultural Bacteriology, *Ann. Report, Wisconsin Agr. Exp. Station*, **21**, p. 368;

Wehmer, Das Laboratorium für technische Bakteriologie an der Techn. Hochschule, Hannover, *Centralbl. f. Bakt.*, II. *Abt.*, **26**, p. 667.

V. SUPPLIES.

Besides small local glass manufacturers, chemists, &c., the following large firms are most important: Paul Altmann, Berlin NW, Luisenstr. 47 (Apparatus and Instruments); Bacteriological Museum and Bureau for the Exchange of Bacterial Cultures at the American Museum of Natural History in New York; Dr. G. Grübler & Co., Leipzig, Liebigstr. 1h (stains, reagents, etc., for microscopic work); Fr. Hugershoff, Leipzig, Carolinenstr. 13 (Instruments, special centrifuges and sedimentation tubes for milk-leucocyte-test); Král's bakteriologisches Museum (Prof. Kraus and Dr. Pribram), Wien IX/3 Zimmermannsgasse 3 (Pure cultures, permanent specimens, microphotograms), London Agents, J. J. Griffin and Co., Kingsway, London, W.C.; F. & M. Lautenschläger, Berlin N 39, Chausseestr. 92 (Apparatus, instruments, etc.); E. Leitz, Optische Werkstätte, Wetzlar (Microscopes); E. Merck, Chem. Fabrik, Darmstadt (Chemicals, used in almost all the large laboratories in the world, especially for comparative experiments);

Dr. Rob. Muencke, Berlin NW 6, Luisenstr. 58 (Apparatus, instruments, etc.); C. Reichert, Wien VIII, Bennogasse 24-26 (Microscopes); Dr. Herm. Rohrbeck Nachf. Berlin NW, Karlstr. 20a (Apparatus, instruments, etc.); F. Sartorius, Göttingen (Balances, Incubators); W. & H. Seibert, Optisches Institut, Wetzlar (Microscopes); Vereinigte Fabriken für Labratoriums-bedarf, G. m. b. H., Berlin N 39, Scharnhorststr. 22 (Apparatus, instruments, etc.); Carl Zeiss, Optische Werkstätte, Jena (Microscopes).

Baird and Tatlock (Ltd.), Cross Street, Hatton Garden, London, E.C. Baird and Tatlock, Renfrew Street, Glasgow; Teviot Place, Edinburgh; High Street, Manchester; and Brownlow Hill, Liverpool. J. J. Griffin and Co., Kingsway, London, W.C. Townson and Mercer, Camomile Street, London, E.C. Reynolds and Branson, Leeds. Philip Harris and Co., Edmund Street, Birmingham. Eimer and Amend, New York, N.Y. A. H. Thomas Co., New York, N.Y. Bausch and Lomb Optical Co., Rochester, N.Y. E. H. Sargent, Chicago, Ill. Emil Greiner Co., New York, N.Y. Scientific Materials Co., Pittsburg, Pa. International Instruments Co., Cambridge, Mass. The Kny Scheerer Co., New York, N.Y.

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